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(54) Title: POLYMORPHISMS WITHIN THE RSK–3 GENE RELATED TO CANCER AND METHODS OF DIAGNOSIS, PROGNOSIS, AND TREATMENT

(57) Abstract

Surprisingly, it has been found that the Rsk–3 gene (p90 ribosomal S6 kinase–3) is mutated in ovarian cancer. The invention provides methods of diagnosis, prognosis and treatment of cancer related to the Rsk–3 gene.

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POLYMORPHISMS WITHIN THE RSK-3 GENE RELATED TO CANCER AND METHODS OF DIAGNOSIS, PROGNOSIS, AND TREATMENT

The present invention relates to cancer and in particular to ovarian cancer.

Cancer is a serious disease and a major killer. Although there have been advances in the diagnosis and treatment of certain cancers in recent years, there is still a need for improvements in diagnosis and treatment.

Cancer is a genetic disease and in most cases involves mutations in one or more genes. There are believed to be around 200,000 genes in the human genome but only a handful of these genes have been shown to be involved in cancer. Although it is surmised that many more genes than have been presently identified will be found to be involved in cancer, progress in this area has remained slow despite the availability of molecular analytical techniques. This may be due to the varied structure and function of genes which have been identified to date which suggests that cancer genes can take many forms and have many different functions.

Ovarian cancer is the most frequent cause of death from gynaecological malignancies in the Western World, with an incidence of 5,000 new cases every year in England and Wales. It is the fourth most common cause of cancer mortality in American women. The majority of patients with epithelial ovarian cancer present at an advanced stage of the disease. Consequently, the 5 year survival rate is only 30% after adequate surgery and chemotherapy despite the introduction of new drugs such as platinum and taxol (Advanced Ovarian Cancer Trialists Group (1991) *BMJ* **303**, 884-893; Ozols (1995) *Semin Oncol.* **22**, 61-66). However, patients who

have stage I disease (confined to the ovaries) do better with the 5 year survival rate being 70%. It is therefore desirable to have techniques to detect the cancer before metastasis to have a significant impact on survival.

Epithelial ovarian cancer constitutes 70-80% of ovarian cancer and encompasses a broad spectrum of lesions, ranging from localized benign tumours and neoplasms of borderline malignant potential to invasive adenocarcinomas. Histologically, the common epithelial ovarian cancers, are classified into several types, that is, serous, mucinous, endometrioid, clear cell, Brenner, mixed epithelial, and undifferentiated tumours. The heterogeneity of histological subtypes reflects the metaplastic potential of the ovarian surface Mullerian epithelium which shares a common embryological origin with the peritoneum and the rest of the uro-genital system. Germ cell, sex cord/stromal tumours and sarcomas represent the remainder of ovarian cancers. The histogenesis and biological characteristics of epithelial ovarian cancer are poorly understood as are the molecular genetic alterations that may contribute to the development of such tumours or their progression. Epidemiological factors related to ovulation seem to be important, whereby ovarian epithelial cells undergo several rounds of division and proliferative growth to heal the wound in the epithelial surface. These lead to the development of epithelial inclusion cysts and frank malignant tumours may arise from them (Fathalla (1971) *Lancet* **2**, 163).

Genetic changes in the tumour are critical for the development of cancer. Many chromosomal regions (chromosomes 3, 5, 6, 8, 11, 13, 17, 18, 22, and X) have been implicated to contain tumour suppressor genes involved

in tumour progression of sporadic ovarian cancer, but only the p53 gene (chromosome arm 17p) has been found to be frequently mutated (Shelling *et al* (1995) *Br. J. Cancer* 72, 521-527). The BRCA1 gene (chromosome arm 17q) and the BRCA2 gene (chromosome arm 13q) isolated in 1994 and 1996 respectively, are mutated in a proportion of patients with familial breast/ovarian cancer (Ford & Easton (1995) *Br. J. Cancer* 72, 805-812). Familial ovarian cancer only accounts for 5-10% of all ovarian tumours. In tumours from patients with sporadic ovarian cancer, only five mutations in the BRCA1 gene and four in the BRCA2 gene have been reported (Takahashi *et al* (1995) *Cancer Res.* 55, 2998-3002; Takahashi *et al* (1996) *Cancer Res.* 56, 2738-2741) suggesting that they are rare in sporadic ovarian cancer. Mutations in the mismatch repair genes have been reported at a frequency of 10% (Tangi *et al* (1996) *Cancer Res.* 56, 2501-2505; Fujita *et al* (1995) *Int. J. Cancer* **64**, 361-366; Orth *et al* (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9495-9499). Thus genes that may be more critical in tumour progression in sporadic ovarian cancer have not yet been fully characterised.

Chromosome 6 has been implicated to contain a putative tumour suppressor gene important in the pathogenesis of ovarian cancer, both by karyotypic analysis and allele loss studies (Shelling *et al* (1995) *Br. J. Cancer* 72, 521-527; Katso *et al* (1997) *Cancer & Metastasis Rev.* 16, 81-107; Saito *et al* (1996) *Cancer Res.* 56, 5586-5589). The analysis of 70 malignant ovarian tumours using cosmids mapping to chromosomal arm 6q and RFLPs (restriction fragment length polymorphisms) to detect LOH (loss of heterozygosity) initially defined the minimal region of allele loss between D6S149 and D6S193 (1.9cM) in one tumour (Saito *et al* (1992)

Cancer Res. 52, 5815-5817) (Fig. 1). A further two studies have shown increased frequency of allele loss on 6q around the same region, though a minimal region was not defined (Wan *et al* (1994) *Int. J. Oncol.* 5, 1043-1048; Orphanos *et al* (1995) *Br. J. Cancer* 71, 666-669). Based on analysis of 56 malignant ovarian tumours it was shown previously that the minimal region of allele loss on 6q27 is between D6S297 and D6S264 (3cM) (Fig. 1). The maximal frequency of allele loss occurred at D6S193 (62%) and D6S297 (52%). Three tumours showed loss of D6S193 only, while retaining flanking markers. The allele loss was observed in all types of epithelial ovarian cancer (Cooke *et al* (1996) *Genes, Chromosomes & Cancer* 15, 223-233).

The following papers also refer to LOH at chromosome 6q 27: Tibiletti *et al* (1996) *Cancer Res.* 56, 4493-4498; Foulker *et al* (1993) *Br J. Cancer* 67, 551-559; Banga *et al* (1997) *Oncogene* 14, 313-321; and Orphanos *et al* (1995) *Br J. Cancer* 71, 290-293.

WO 96/05306, WO 96/05307 and WO 96/05308 relate to methods and materials used to isolate and detect a human breast and ovarian cancer predisposing gene (BRCA1), some mutant alleles of which are alleged to cause susceptibility to cancer, in particular breast and ovarian cancer.

Surprisingly, out of the plethora of genes in the genome, it has now been found that the p90 ribosomal S6 kinase-3 gene (Rsk-3), which lies outside of the minimal region identified previously, is frequently mutated in sporadic epithelial ovarian cancer, particularly in malignant and borderline epithelial ovarian tumours, and it is believed that it is this gene which is

involved in ovarian cancer. As is described in more detail below, Rsk-3 is related to two other genes, Rsk-1 and Rsk-2, neither of which have been implicated in cancer. Certain mutations in malignant ovarian cancer cell lines renders the kinase constitutively active. Mutations in p90 ribosomal S6 kinase-3 (Rsk-3) may represent one of the earliest genetic events in the development of ovarian cancer and, therefore, it may be particularly important to identify changes in this gene.

A first aspect of the invention provides a method for determining the susceptibility of a patient to cancer comprising the steps of (i) obtaining a sample containing nucleic acid from the patient; and (ii) contacting the said nucleic acid with a nucleic acid which hybridises selectively to the Rsk-3 gene, or a mutant allele thereof, or a nucleic acid which hybridises selectively to Rsk-3 cDNA, or a mutant allele thereof, or their complement.

A second aspect of the invention provides a method of diagnosing cancer in a patient comprising the steps of (i) obtaining a sample containing nucleic acid from the patient; and (ii) contacting the said nucleic acid with a nucleic acid which hybridises selectively to the Rsk-3 gene, or a mutant allele thereof, or a nucleic acid which hybridises selectively to Rsk-3 cDNA, or a mutant allele thereof, or their complement.

A third aspect of the invention provides a method of predicting the relative prospects of a particular outcome of a cancer in a patient comprising the steps of (i) obtaining a sample containing nucleic acid from the patient; and (ii) contacting the said nucleic acid with a nucleic acid which hybridises selectively to the Rsk-3 gene, or a mutant allele thereof, or a

nucleic acid which hybridises selectively to Rsk-3 cDNA, or a mutant allele thereof, or their complement.

The Rsk-3 gene is located within the two, overlapping P1-artificial chromosome (PAC) clones 168L15 and 427A4. These PACs overlap as judged by "fingerprinting" with restriction enzymes. Both of these PACs are publicly available from the Human Genome Mapping Resource Centre, Hinxton Hall, Hinxton, Cambridgeshire CB10 1RQ, UK or the Sanger Centre, Wellcome Trust Genome Campus, Hinxton Hall, Hinxton, Cambridge CB10 1SA, UK, and the Rothwell Park Cancer Institute, Human Genetics Department, Elm and Carlton Streets, Buffalo, New York, 14263-0001, USA. PAC clone 427A4 is from the RPCI3 human PAC library (human, male). PAC clone 168L15 is from the RPCI-1 library (human, male). PAC clone 168L15 has been deposited under the Budapest Treaty at the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen, AB24 3RY, Scotland, UK under accession number NCIMB 40914 as an *Escherichia coli* clone.

Although PAC clone 497J21 does not contain any of Rsk-3's exons, it may contain certain regulatory regions of the Rsk-3 gene. PAC 497J21 is available from the Human Genome Mapping Resource Centre, as above.

The complete nucleotide sequence of all of the PAC clones is publicly available from the GenBank/EMBL databank. The sequence in PAC clone 427A4 is held under Accession No Z98049. The sequence of PAC clone 168L15 is held under Accession No AL022069. The sequence of PAC clone 497J21 is held under Accession No AL023775 (see Figure 16).

It will readily be appreciated by the skilled person that the Rsk-3 gene or parts thereof may readily be obtained from other suitable human gene libraries, such as standard cosmid, or yeast artificial chromosome (YAC) or P1-artificial chromosome (PAC) libraries using the aforementioned PAC clones, or fragments thereof, as probes. Similarly, an Rsk-3 cDNA may be used as a probe to identify all or parts of the Rsk-3 gene.

An Rsk-3 cDNA is described in Zhao *et al* (1995) *Mol. Cell. Biol.* **15**, 4353-4363, incorporated herein by reference, and this cDNA sequence is publicly available from the EMBL Genbank data library under Accession No X85106. This sequence is also shown in Figure 6. In any event, an Rsk-3 cDNA may be readily obtained from a human cDNA library using well known techniques and portions of the PAC clones 168L15 or 427A4, or portions of the Rsk-3 cDNA sequence shown in Figure 6, as a probe. A suitable human cDNA library is one prepared from mRNA isolated from a human ovary or human ovarian tissue or from a human ovarian cell line. Once an Rsk-3 cDNA or gene or fragment thereof has been identified as said, its nucleotide sequence may readily be determined, for example using Sanger dideoxy sequencing or other methods well known in the art.

It will be appreciated (and as is described in more detail in the Examples) that the Rsk-3 gene may exist as a "wild-type" gene or it may exist as mutant alleles which differ in sequence to the wild-type gene. By "mutant alleles" is included not only sequences which lead to changes in function or expression of the Rsk-3 polypeptide, but allelic variants (or polymorphisms) which have no or only minor effect on the function or expression of the Rsk-3 polypeptide. Thus, the nucleic acids which

selectively hybridise in the methods of the invention include those that selectively hybridise to the wild-type Rsk-3 gene sequence or to the wild-type Rsk-3 cDNA sequence (or mRNA sequence) as well as those which selectively hybridise to mutant alleles thereof. Also, it will readily be appreciated that, as is described in more detail herein, the skilled person can readily identify mutant alleles of the Rsk-3 gene and polymorphisms thereof. By "change in expression of the Rsk-3 polypeptide" is included any changes in the Rsk-3 gene which lead to changes in expression of the Rsk-3 polypeptide. For example, changes in the transcription of the Rsk-3 gene will lead to changes in the expression of the Rsk-3 polypeptide. Similarly, changes in the translation of Rsk-3 mRNA will lead to changes in the expression of the Rsk-3 polypeptide.

Changes in the function of the Rsk-3 polypeptide include, but are not limited to, changes in enzyme activity (such as K_m or k_{cat}), changes in substrate specificity and changes which alter the response by Rsk-3 to proteins which regulate its function.

It will be appreciated that the nucleic acids which are useful in the method of the invention may readily be defined as those which selectively hybridise to the human-derived DNA of PAC clone 168L15 or PAC clone 427A4, or which selectively hybridise to Rsk-3 cDNA, or a mutant allele thereof, or their complement. In addition, the methods of the invention include the use of a nucleic acid which selectively hybridises to the Rsk-3 gene or cDNA, or mutant alleles thereof whatever the source of the gene or cDNA.

By "selectively hybridising" is meant that the nucleic acid has sufficient nucleotide sequence similarity with the said human DNA or cDNA that it can hybridise under moderately or highly stringent conditions. As is well known in the art, the stringency of nucleic acid hybridization depends on factors such as length of nucleic acid over which hybridisation occurs, degree of identity of the hybridizing sequences and on factors such as temperature, ionic strength and CG or AT content of the sequence. Thus, any nucleic acid which is capable of selectively hybridising as said is useful in the practice of the invention.

Nucleic acids which can selectively hybridise to the said human DNA or cDNA include nucleic acids which have >95% sequence identity, preferably those with >98%, more preferably those with >99% sequence identity, over at least a portion of the nucleic acid with the said human DNA or cDNA. As is well known, human genes usually contain introns such that, for example, a mRNA or cDNA derived from a gene within the said human DNA would not match perfectly along its entire length with the said human DNA but would nevertheless be a nucleic acid capable of selectively hybridising to the said human DNA. Thus, the invention specifically includes nucleic acids which selectively hybridise to an Rsk-3 cDNA but may not hybridise to an Rsk-3 gene, or *vice versa*. For example, nucleic acids which span the intron-exon boundaries of the Rsk-3 gene may not be able to selectively hybridise to the Rsk-3 cDNA.

Typical moderately or highly stringent hybridisation conditions which lead to selective hybridisation are known in the art, for example those described in *Molecular Cloning, a laboratory manual*, 2nd edition,

Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by reference.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe nucleic acid is ≥ 500 bases or base pairs is:

6 x SSC (saline sodium citrate)
0.5% sodium dodecyl sulphate (SDS)
100 µg/ml denatured, fragmented salmon sperm DNA

The hybridisation is performed at 68°C. The nylon membrane, with the nucleic acid immobilised, may be washed at 68°C in 1 x SSC or, for high stringency, 0.1 x SSC.

20 x SSC may be prepared in the following way. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 litre with H_2O. Dispense into aliquots. Sterilize by autoclaving.

An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe is an oligonucleotide of between 15 and 50 bases is:

3.0 M trimethylammonium chloride (TMACl)
0.01 M sodium phosphate (pH 6.8)
1 mm EDTA (pH 7.6)
0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA
0.1% nonfat dried milk

The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. T_i is the irreversible melting temperature of the hybrid formed between the probe and its target sequence. Jacobs *et al* (1988) *Nucl. Acids Res.* **16**, 4637 discusses the determination of T_is. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48-50°C; for 19-mers, it is 55-57°C; and for 20-mers, it is 58-66°C.

By "nucleic acid which selectively hybridises" is also included nucleic acids which will amplify DNA from the said region of human DNA by any of the well known amplification systems such as those described in more detail below, in particular the polymerase chain reaction (PCR). Suitable conditions for PCR amplification include amplification in a suitable 1 x amplification buffer:

10 x amplification buffer is 500 mM KCl; 100 mM Tris.Cl (pH 8.3 at room temperature); 15 mM $MgCl_2$; 0.1% gelatin.

A suitable denaturing agent or procedure (such as heating to 95°C) is used in order to separate the strands of double-stranded DNA.

Suitably, the annealing part of the amplification is between 37°C and 60°C, preferably 50°C.

Although the nucleic acid which is useful in the methods of the invention may be RNA or DNA, DNA is preferred. Although the nucleic acid which is useful in the methods of the invention may be double-stranded or single-stranded, single-stranded nucleic acid is preferred under some circumstances such as in nucleic acid amplification reactions.

The nucleic acid which is useful in the methods of the invention may be very large, such as 100 kb, if it is double stranded. For example, such large nucleic acids are useful as a template for making probes for use in FISH (fluorescence *in situ* hybridization) analysis. Typically, the labelled probes used in FISH are generally made by nick-translation or random priming from a genomic clone (such as an insert in a suitable PAC clone). Once made these probes are around 50-1000 nucleotides in length. The human DNA insert of PAC clone 427A4, which may be a useful probe in its own right, is 149 kb but is more preferably used as a template for nick-translation or random primer extension as described above. However, for certain diagnostic, probing or amplifying purposes, it is preferred if the nucleic acid has fewer than 10 000, more preferably fewer than 1000, more preferably still from 10 to 100, and in further preference from 15 to 30 base pairs (if the nucleic acid is double-stranded) or bases (if the nucleic acid is single stranded). As is described more fully below, single-stranded DNA primers, suitable for use in a polymerase chain reaction, are particularly preferred.

The nucleic acid for use in the methods of the invention is a nucleic acid capable of hybridising to the Rsk-3 gene. Fragments and variants of this gene, and cDNAs derivable from the mRNA encoded by the gene are also preferred nucleic acids for use in the methods of the invention.

Clearly nucleic acids which selectively hybridise to the gene itself or variants thereof are particularly useful. Fragments of the gene are preferred for use in the method of the invention. Fragments may be made by enzymatic or chemical degradation of a larger fragment, or may be chemically synthesised. By "gene" is included not only the introns and exons but also regulatory regions associated with, and physically close to, the introns and exons, particularly those 5′ to the 5′-most exon. By "physically close" is meant within 50 kb, preferably within 10 kb, more preferably within 5 kb and still more preferably within 2 kb. It is believed that the basic promoter and regulatory elements of the Rsk-3 gene probably lie up to 200-400 base pairs of the transcriptional start site or start of the coding region. However, tissue specific or inducible elements may be 50 kb in either direction of the coding regions (exons) or may be in the introns. Such elements of the Rsk-3 gene may be identified or located by DNAse hypersensitivity sites (detected on Southern blots) which indicate sites of regulatory protein binding. Alternatively, reporter constructs may be generated using the upstream genomic DNA (ie upstream of the 5′-most exon) and, for example, β-galactosidase as a reporter enzyme. Serial deletions and footprinting techniques may also be used to identify the regulatory regions.

By "fragment" of a gene is included any portion of the gene of at least 15 nucleotides in length (whether single stranded or double stranded) but more preferably the fragment is at least 20 nucleotides in length, most preferably at least 50 nucleotides in length and may be at least 100 nucleotides in length or may be at least 500 nucleotides in length.

Preferably the fragment is no more than 50 kb and, more preferably, no more than 100 kb.

By "variant" of a gene is included specifically a cDNA, whether partial or full length, or whether copied from any splice variants of mRNA. We also include specifically a nucleic acid wherein, compared to the natural gene, nucleotide substitutions (including inversions), insertions and deletions are present whether in the gene or a fragment thereof or in a cDNA. Both variants and fragments will be selected according to their intended purposes; for probing, amplifying or diagnostic purposes, shorter fragments but with a greater degree of sequence identity (eg at least 80%, 90%, 95% or 99%) will generally be required.

It is particularly preferred if the nucleic acid for use in the methods of the invention is an oligonucleotide primer which can be used to amplify a portion of the gene.

The Rsk-3 gene and Rsk-3 cDNA are similar to, but distinct from, the Rsk-1 gene and cDNA, and the Rsk-2 gene and cDNA. Preferred nucleic acids for use in the invention are those that selectively hybridise to the Rsk-3 gene or cDNA and do not hybridise to the Rsk-1 gene or cDNA and do not hybridise to the Rsk-2 gene or cDNA. Such selectively hybridising nucleic acids can be readily obtained, for example, by reference to whether or not they hybridise to the Rsk-1 gene or Rsk-2 gene, or whether they hybridise to the Rsk-1 cDNA or Rsk-2 cDNA. The Rsk-2 gene is located at Xp22.2 and the Rsk-1 gene is located on chromosome 3 at 3p. The Rsk-1 cDNA sequence is shown in Figure 15 and the Rsk-2 cDNA sequence is shown in Figure 14.

The methods are suitable in respect of any cancer but it is preferred if the cancer is cancer of the ovary, breast, vulva, vagina or cervix or if the cancer is lymphoma. The methods are particularly suitable in respect of cancer of the ovary or breast, or lymphoma; and the methods are most suitable in respect of ovarian cancer. It will be appreciated that the methods of the invention include methods of prognosis and methods which aid diagnosis. It will also be appreciated that the methods of the invention are useful to the physician or surgeon in determining a course of management or treatment of the patient.

Although it is believed that any sample containing nucleic acid derived from the patient is useful in the methods of the invention, since mutations in the Rsk-3 gene may occur in familial cancers and not just sporadic cancers, it is, however, preferred if the nucleic acid is derived from a sample of the tissue in which cancer is suspected or in which cancer may be or has been found. For example, if the tissue in which cancer is suspected or in which cancer may be or has been found is ovary, it is preferred if the sample containing nucleic acid is derived from the ovary of the patient. Samples of ovary may be obtained by surgical excision, laproscopy and biopsy, endoscopy and biopsy, and image-guided biopsy. The image may be generated by ultrasound or technetium-99-labelled antibodies or antibody fragments which bind or locate selectively at the ovary. The well known monoclonal antibody HMFG1 is a suitable antibody for imaging ovarian cancer. Ascites/peritoneal cavity fluid, and peritoneal samples, may be obtained by surgery or laproscopy. Similarly, if the tissue in which cancer is suspected or in which cancer may be or has been found is breast, it is preferred if the sample containing nucleic acid is

derived from the breast of the patient; and so on. Breast samples may be obtained by excision, "true cut" biopsies, needle biopsy, nipple aspirate or image-guided biopsy.

Other samples in which it may be beneficial to analyse Rsk-3 include lymph nodes, blood, serum and potential or actual sites of metastasis, for example bone.

The sample may be directly derived from the patient, for example, by biopsy of the tissue, or it may be derived from the patient from a site remote from the tissue, for example because cells from the tissue have migrated from the tissue to other parts of the body. Alternatively, the sample may be indirectly derived from the patient in the sense that, for example, the tissue or cells therefrom may be cultivated *in vitro*, or cultivated in a xenograft model; or the nucleic acid sample may be one which has been replicated (whether *in vitro* or *in vivo*) from nucleic acid from the original source from the patient. Thus, although the nucleic acid derived from the patient may have been physically within the patient, it may alternatively have been copied from nucleic acid which was physically within the patient. The tumour tissue may be taken from the primary tumour or from metastases.

It will be appreciated that a useful method of the invention includes the analysis of mutations in, or the detection of the presence or absence of, the Rsk-3 gene in any suitable sample. The sample may suitably be a freshly-obtained sample from the patient, or the sample may be an historic sample, for example a sample held in a library of samples.

Certain mutations in the Rsk-3 gene which are associated with cancer are described in the Examples.

Conveniently, the nucleic acid capable of selectively hybridising to the said human DNA and which is used in the methods of the invention further comprises a detectable label.

By "detectable label" is included any convenient radioactive label such as ^{32}P, ^{33}P or ^{35}S which can readily be incorporated into a nucleic acid molecule using well known methods; any convenient fluorescent or chemiluminescent label which can readily be incorporated into a nucleic acid is also included. In addition the term "detectable label" also includes a moiety which can be detected by virtue of binding to another moiety (such as biotin which can be detected by binding to streptavidin); and a moiety, such as an enzyme, which can be detected by virtue of its ability to convert a colourless compound into a coloured compound, or *vice versa* (for example, alkaline phosphatase can convert colourless o-nitrophenylphosphate into coloured *o*-nitrophenol). Conveniently, the nucleic acid probe may occupy a certain position in a fixed assay and whether the nucleic acid hybridises to the said region of human DNA can be determined by reference to the position of hybridisation in the fixed assay. The detectable label may also be a fluorophore-quencher pair as described in Tyagi & Kramer (1996) *Nature Biotechnology* **14**, 303-308.

It will be appreciated that the aforementioned methods may be used for presymptomatic screening of a patient who is in a risk group for cancer. High risk patients for screening include patients over 50 years of age or patients who carry a gene resulting in increased susceptibility (eg

predisposing versions of BRCA1, BRCA2 or p53); patients with a family history of breast/ovarian cancer; patients with affected siblings; nulliparous women; and women who have a long interval between menarche and menopause. Similarly, the methods may be used for the pathological classification of tumours such as ovarian tumours.

Conveniently, in the methods of the first, second and third aspects of the invention the nucleic acid which is capable of the said selective hybridisation (whether labelled with a detectable label or not) is contacted with a nucleic acid derived from the patient under hybridising conditions. Suitable hybridising conditions include those described above.

It is preferred that if the sample containing nucleic acid derived from the patient is not a substantially pure sample of the tissue or cell type in question that the sample is enriched for the said tissue or cells. For example, enrichment for ovarian cells in a sample such as a blood sample may be achieved using, for example, cell sorting methods such as fluorescent activated cell sorting (FACS) using an ovary cell-selective antibody, or at least an antibody which is selective for an epithelial cell. For example, Cam 5.2, anticytokeratin 7/8, from Becton Dickinson, 2350 Qume Drive, San Jose, California, USA, may be useful. The source of the said sample also includes biopsy material as discussed above and tumour samples, also including fixed paraffin mounted specimens as well as fresh or frozen tissue. The nucleic acid sample from the patient may be processed prior to contact with the nucleic acid which selectively hybridises to Rsk-3. For example, the nucleic acid sample from the patient may be treated by selective amplification, reverse transcription,

immobilisation (such as sequence specific immobilisation), or incorporation of a detectable marker.

It is particularly preferred if the methods of the invention include the determination of mutations in, or the detection of the presence or absence of, the Rsk-3 gene.

The methods of the first, second and third aspects of the invention may involve sequencing of DNA at one or more of the relevant positions within the relevant region, including direct sequencing; direct sequencing of PCR-amplified exons; differential hybridisation of an oligonucleotide probe designed to hybridise at the relevant positions within the relevant region (conveniently this uses immobilised oligonucleotide probes in, so-called, "chip" systems which are well known in the art); denaturing gel electrophoresis following digestion with an appropriate restriction enzyme, preferably following amplification of the relevant DNA regions; S1 nuclease sequence analysis; non-denaturing gel electrophoresis, preferably following amplification of the relevant DNA regions; conventional RFLP (restriction fragment length polymorphism) assays; heteroduplex analysis; selective DNA amplification using oligonucleotides; fluorescent *in-situ* hybridisation (FISH) of interphase chromosomes; ARMS-PCR (Amplification Refractory Mutation System-PCR) for specific mutations; cleavage at mismatch sites in hybridised nucleic acids (the cleavage being chemical or enzymic); SSCP single strand conformational polymorphism or DGGE (discontinuous or denaturing gradient gel electrophoresis); analysis to detect mismatch in annealed normal/mutant PCR-amplified DNA; and protein truncation assay (translation and transcription of exons - if a mutation introduces a stop codon a truncated protein product will

result). Other methods may be employed such as detecting changes in the secondary structure of single-stranded DNA resulting from changes in the primary sequence, for example, using the cleavase I enzyme. This system is commercially available from GibcoBRL, Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, Scotland.

It will be appreciated that the methods of the invention may also be carried out on "DNA chips". Such "chips" are described in US 5,445,934 (Affymetrix; probe arrays), WO 96/31622 (Oxford; probe array plus ligase or polymerase extension), and WO 95/22058 (Affymax; fluorescently marked targets bind to oligomer substrate, and location in array detected); all of these are incorporated herein by reference.

Detailed methods of mutation detection are described in "Laboratory Protocols for Mutation Detection" 1996, ed. Landegren, Oxford University Press on behalf of HUGO (Human Genome Organisation).

It is preferred if RFLP is used for the detection of fairly large (≥ 500bp) deletions or insertions. Southern blots may be used for this method of the invention.

PCR amplification of smaller regions (maximum 300bp) to detect small changes greater than 3-4 bp insertions or deletions may be preferred. Amplified sequence may be analysed on a sequencing gel, and small changes (minimum size 3-4 bp) can be visualised. Suitable primers are designed as herein described.

In addition, using either Southern blot analysis or PCR restriction enzyme variant sites may be detected. For example, for analysing variant sites in genomic DNA restriction enzyme digestion, gel electrophoresis, Southern blotting, and hybridisation specific probe (for example any suitable fragment derived from the Rsk-3 cDNA or gene).

For example, for analysing variant sites using PCR DNA amplification, restriction enzyme digestion, gel detection by ethidium bromide, silver staining or incorporation of radionucleotide or fluorescent primer in the PCR.

Other suitable methods include the development of allele specific oligonucleotides (ASOs) for specific mutational events. Similar methods are used on RNA and cDNA for the suitable tissue, such as ovarian or breast tissue.

Whilst it is useful to detect mutations in any part of the Rsk-3 gene, it is preferred if the mutations are detected in the exons of the gene and it is further preferred if the mutations are ones which change the coding sense. Mutations have been found in several exons of the Rsk-3 gene which are associated with ovarian cancer as is described in more detail in the Examples. The detection of these mutations is a preferred aspect of the invention. Similarly, the invention also includes probes and primers and other means for detecting the specific mutations identified in the Examples, all of which can be designed, made and used by methods well known to the skilled person.

The methods of the invention also include checking for loss-of-heterozygosity (LOH; shows one copy lost). LOH may be a sufficient marker for diagnosis; looking for mutation/loss of the second allele may not be necessary. LOH of the gene may be detected using polymorphisms in the coding sequence, and introns, of the gene. Polymorphisms in the exons of Rsk-3 have been identified (eg 1485: ACA or ACG; 855: GCA or GCG). LOH in a tumour cell, from whatever source, compared to blood is useful as a diagnostic tool, eg it may show that the tumour has progressed and requires more stringent treatment.

Particularly preferred nucleic acids for use in the aforementioned methods of the invention are those selected from the group consisting of primers suitable for amplifying nucleic acid.

Suitably, the primers are selected from the group consisting of primers which hybridise to the nucleotide sequences shown in any of the Figures which show Rsk-3 gene or cDNA sequences. It is particularly preferred if the primers hybridise to the introns of the Rsk-3 gene or if the primers are ones which will prime synthesis of DNA from the Rsk-3 gene or cDNA but not from the Rsk-1 or Rsk-2 gene or cDNA. Particularly preferred primers are shown in Figure 13.

Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki *et al* (1988) *Science* **239**, 487-491) are preferred. Suitable PCR primers may have the following properties:

It is well known that the sequence at the 5′ end of the oligonucleotide need not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3′ ends, as this feature may promote the formation of an artifactual product called "primer dimer". When the 3′ ends of the two primers hybridize, they form a "primed template" complex, and primer extension results in a short duplex product called "primer dimer".

Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with no long stretches of any one base. The classical melting temperature calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple T_m calculations.

Optimum annealing temperatures may be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step and the hybrid will be stabilized. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1-μM range.

Any of the nucleic acid amplification protocols can be used in the method of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. Also, NASBA (nucleic acid sequence based amplification), also called 3SR, can be used as described in Compton

(1991) *Nature* **350**, 91-92 and *AIDS* (1993), Vol 7 (Suppl 2), S108 or SDA (strand displacement amplification) can be used as described in Walker *et al* (1992) *Nucl. Acids Res.* **20**, 1691-1696. The polymerase chain reaction is particularly preferred because of its simplicity.

When a pair of suitable nucleic acids of the invention are used in a PCR it is convenient to detect the product by gel electrophoresis and ethidium bromide staining. As an alternative to detecting the product of DNA amplification using agarose gel electrophoresis and ethidium bromide staining of the DNA, it is convenient to use a labelled oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by a PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The oligonucleotide probe is preferably between 10 and 50 nucleotides long, more preferably between 15 and 30 nucleotides long. The probe may be labelled with a radionuclide such as ^{32}P, ^{33}P and ^{35}S using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991) "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* **195**, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* **15**, 152-157.

PCR products can also be detected using a probe which may have a fluorophore-quencher pair or may be attached to a solid support or may have a biotin tag or they may be detected using a combination of a capture probe and a detector probe.

Fluorophore-quencher pairs are particularly suited to quantitative measurements of PCR reactions (eg RT-PCR). Fluorescence polarisation using a suitable probe may also be used to detect PCR products.

Oligonucleotide primers can be synthesised using methods well known in the art, for example using solid-phase phosphoramidite chemistry.

The present invention provides the use of a nucleic acid which selectively hybridises to the human-derived DNA of any of the PACs 168L15 or 427A4 or to the Rsk-3 gene, or a mutant allele thereof, or a nucleic acid which selectively hybridises to Rsk-3 cDNA or a mutant allele thereof, or their complement in a method of diagnosing cancer or prognosing cancer or determining susceptibility to cancer; or in the manufacture of a reagent for carrying out these methods.

Also, the present invention provides a method of determining the presence or absence, or mutation in, the said Rsk-3 gene. Preferably, the method uses a suitable sample from a patient.

The methods of the invention include the detection of mutations in the Rsk-3 gene.

The methods of the invention may make use of a difference in restriction enzyme cleavage sites caused by mutation. A non-denaturing gel may be used to detect differing lengths of fragments resulting from digestion with an appropriate restriction enzyme.

An "appropriate restriction enzyme" is one which will recognise and cut the wild-type sequence and not the mutated sequence or *vice versa*. The sequence which is recognised and cut by the restriction enzyme (or not, as the case may be) can be present as a consequence of the mutation or it can be introduced into the normal or mutant allele using mismatched oligonucleotides in the PCR reaction. It is convenient if the enzyme cuts DNA only infrequently, in other words if it recognises a sequence which occurs only rarely.

In another method, a pair of PCR primers are used which match (ie hybridise to) either the wild-type genotype or the mutant genotype but not both. Whether amplified DNA is produced will then indicate the wild-type or mutant genotype (and hence phenotype). However, this method relies partly on a negative result (ie the absence of amplified DNA) which could be due to a technical failure. It therefore may be less reliable and/or requires additional control experiments.

A preferable method employs similar PCR primers but, as well as hybridising to only one of the wild-type or mutant sequences, they introduce a restriction site which is not otherwise there in either the wild-type or mutant sequences.

The nucleic acids which selectively hybridise to the Rsk-3 gene or cDNA, or which selectively hybridise to the PAC clones 168L15 or 427A4 are useful for a number of purposes. They can be used in Southern hybridization to genomic DNA and in the RNase protection method for detecting point mutations already discussed above. The probes can be used to detect PCR amplification products. They may also be used to

detect mismatches with the Rsk-3 gene or mRNA in a sample using other techniques. Mismatches can be detected using either enzymes (eg S1 nuclease or resolvase), chemicals (eg hydroxylamine or osmium tetroxide and piperidine), or changes in electrophoretic mobility of mismatched hybrids as compared to totally matched hybrids. These techniques are known in the art. Generally, the probes are complementary to the Rsk-3 gene coding sequences, although probes to certain introns are also contemplated. A battery of nucleic acid probes may be used to compose a kit for detecting loss of or mutation in the wild-type Rsk-3 gene. The kit allows for hybridization to the entire Rsk-3 gene. The probes may overlap with each other or be contiguous.

If a riboprobe is used to detect mismatches with mRNA, it is complementary to the mRNA of the human Rsk-3 gene. The riboprobe thus is an anti-sense probe in that it does not code for the protein encoded by the Rsk-3 gene because it is of the opposite polarity to the sense strand. The riboprobe generally will be labelled, for example, radioactively labelled which can be accomplished by any means known in the art. If the riboprobe is used to detect mismatches with DNA it can be of either polarity, sense or anti-sense. Similarly, DNA probes also may be used to detect mismatches.

Nucleic acid probes may also be complementary to mutant alleles of the Rsk-3 gene. These are useful to detect similar mutations in other patients on the basis of hybridization rather than mismatches. As mentioned above, the Rsk-3 gene probes can also be used in Southern hybridizations to genomic DNA to detect gross chromosomal changes such as deletions and insertions.

According to the diagnostic and prognostic method of the present invention, loss of, or modification of, the wild-type gene function may be detected. The loss may be due to either insertional, deletional or point mutational events. If only a single allele is mutated, an early neoplastic state may be indicated. However, if both alleles are mutated then a malignant state is indicated or an increased probability of malignancy is indicated. The finding of such mutations thus provides both diagnostic and prognostic information. An Rsk-3 gene allele which is not deleted (eg that on the sister chromosome to a chromosome carrying a gene deletion) can be screened for other mutations, such as insertions, small deletions, and point mutations. We believe that detecting a mutation in a single copy (allele) of the gene is useful. Loss of the second allele may be necessary for carcinogenesis. If the second copy was lost routinely by a gross mechanism, this could be a useful event to detect. Some mutations of the gene may have a dominant negative effect on the remaining allele. Mutations leading to non-functional gene products may also lead to a malignant state or an increased probability of malignancy. Mutational events (such as point mutations, deletions, insertions and the like) may occur in regulatory regions, such as in the promoter of the gene, leading to loss or diminution of expression of the mRNA. Point mutations may also abolish proper RNA processing, leading to loss of or alteration in the expression of the Rsk-3 gene product or to the Rsk-3 polypepide being non-functional or having an altered expression. It is preferred if the amount of Rsk-3 mRNA in a test sample is quantified and compared to that present in a control sample. It is also preferred if the splicing patterns or structure of Rsk-3 mRNA in a test sample is determined and compared

to that present in a control sample. However, the detection of altered Rsk-3 expression is less preferred.

The gene has two alleles, and it will be appreciated that alterations to both alleles may have a greater effect on cell behaviour than alteration to one. It is expected that at least one mutant allele has mutations which result in an altered coding sequence. Modifications to the second allele, other than to the coding sequence, may include total or partial gene deletion, and loss or mutation of regulatory regions.

The amount of Rsk-3 mRNA is suitably determined per unit mass of sample tissue or per unit number of sample cells and compared this to the unit mass of known normal tissue or per unit number of normal cells. RNA may be quantitated using, for example, northern blotting or quantitative RT-PCR.

The invention also includes the following methods: *in vitro* transcription and translation of Rsk-3 gene to identify truncated gene products, or altered properties such as substrate binding; immunohistochemistry of tissue sections to identify cells in which expression of the protein is reduced/lost, or its distribution is altered within cells or on their surface; and the use of RT-PCR using random primers, prior to detection of mutations in the region as described above. It is preferred if altered distribution of the Rsk-3 polypeptide is screened for.

A further aspect of the invention provides a system (or it could also be termed a kit of parts) for detecting the presence or absence of, or mutation in, the relevant region of human DNA, the system comprising a nucleic

acid capable of selectively hybridising to the relevant region of human DNA and a nucleoside triphosphate or deoxynucleoside triphosphate or derivative thereof. Preferred nucleic acids capable of selectively hybridising to the relevant region of human DNA are the same as those preferred above.

The "relevant region of human DNA" includes the Rsk-3 gene, the Rsk-3 cDNA and the human-derived DNA present in PAC clones 168L15 or 427A4. Preferably, the relevant region of human DNA is the Rsk-3 gene as herein defined.

By "mutation" is included insertions, substitutions and deletions.

By "nucleoside triphosphate or deoxynucleoside triphosphate or derivative thereof" is included any naturally occurring nucleoside triphosphate or deoxynucleoside triophosphate such as ATP, GTP, CTP, and UTP, dATP dGTP, dCTP, TTP as well as non-naturally derivatives such as those that include a phosphorothioate linkage (for example αS derivatives).

Conveniently the nucleoside triphosphate or deoxynucleoside triphosphosphate is radioactively labelled or derivative thereof, for example with ^{32}P, ^{33}P or ^{35}S, or is fluorescently labelled or labelled with a chemiluminescence compound or with digoxygenin.

Conveniently deoxynucleotides are at a concentration suitable for dilution to use in a PCR.

Thus, the invention includes a kit of parts which includes a nucleic acid capable of selectively hybridising to the said relevant region of human DNA and means for detecting the presence or absence of, or a mutation in, the said region. Means for detecting the presence or absence of, or a mutation in, the said region include, for example, a diagnostic restriction enzyme or a mutant-specific nucleic acid probe or the like.

A further aspect of the invention provides a system for detecting the presence or absence of, or mutation in, the relevant region of DNA, the system comprising a nucleic acid which selectively hybridises to the relevant region of human DNA and a nucleic acid modifying enzyme. Preferred nucleic acids capable of selectively hybridising to the relevant region of human DNA are the same as those preferred above.

By "mutation" is included insertions, substitutions (including transversions) and deletions.

By "nucleic acid modifying enzyme" is included any enzyme capable of modifying an RNA or DNA molecule.

Preferred enzymes are selected from the group consisting of DNA polymerases, DNA ligases, polynucleotide kinases or restriction endonucleases. A particularly preferred enzyme is a thermostable DNA polymerase such as *Taq* DNA polymerase. Nucleases such as Cleavase I which recognise secondary structure, for example mismatches, may also be useful.

Detecting mutations in the gene will be useful for determining the appropriate treatment for a patient, eg Rsk-3 gene therapy (see below). Detecting mutations in the gene may be useful to identify a subset of patients whose tumours have this shared characteristic, and can be analysed as a group for prognosis or response to various therapies.

As the gene appears to be an early event, detection of mutations in it may be useful for screening and diagnosis of ovarian cancers.

Mutations in the gene may be related to response or resistance to certain treatments, this may be investigated using cell lines with known sensitivity to various therapies, or by clinical correlation studies.

It is possible that the gene would be used as part of a panel of markers and tests, which the combined results of would direct therapy. Detecting mutations in the gene may be useful for monitoring disease spread and load.

Analysis of the gene may be useful for differential diagnosis in the case where mutations in the gene are common in one tumour, but not another. For example, secondary tumours of gastrointestinal origin are frequently found in the ovaries and are difficult to distinguish from tumours of true ovarian origin.

A further aspect of the invention provides a method for determining the susceptibility of a patient to cancer comprising the steps of (i) obtaining a sample containing protein derived from the patient; and (ii) determining the relative amount, or the intracellular location, or physical form, of the

Rsk-3 polypeptide, or the relative activity of, or change in activity of, or altered activity of, the Rsk-3 polypeptide.

A still further aspect of the invention provides a method of diagnosing cancer in a patient comprising the steps of (i) obtaining a sample containing protein derived from the patient; and (ii) determining the relative amount, or the intracellular location, or physical form, of the Rsk-3 polypeptide, or the relative activity of, or change in activity of, or altered activity of, the Rsk-3 polypeptide.

A yet still further aspect of the invention provides a method of predicting the relative prospects of a particular outcome of a cancer in a patient comprising the steps of (i) obtaining a sample containing protein derived from the patient; and (ii) determining the relative amount, or the intracellular location, or physical form of the Rsk-3 polypeptide, or the relative activity of, or change in activity of, or altered activity of, the Rsk-3 polypeptide.

The methods of the invention also include the measurement and detection of the Rsk-3 polypeptide or mutants thereof in test samples and their comparison in a control sample. It may also be useful to detect altered activity of the polypeptide. This may be done by detecting increased or constitutive Rsk-3 kinase activity (which can be measured using S6 or Kemptide peptides, as described in more detail below, or altered activity of the polypeptide may be detected by altered substrate specificity, including increased phosphorylation of certain substrates, or decreased phosphorylation of other substrates. Rsk-3 may be a substrate for its own kinase activity.

The sample containing protein derived from the patient is conveniently a sample of the tissue in which cancer is suspected or in which cancer may be or has been found. These methods may be used for any cancer, but they are particularly suitable in respect of cancer of the ovary, breast, vulva, vagina or cervix, or lymphoma; the methods are especially suitable in respect of cancer of the ovary or breast, or lymphoma; the methods are most suitable in respect of ovarian cancer. Methods of obtaining suitable samples are described in relation to earlier methods.

The methods of the invention involving detection of the Rsk-3 polypeptide are particularly useful in relation to historical samples such as those containing paraffin-embedded sections of tumour samples.

The relative amount of, or the intracellular location of, or the physical form of, the Rsk-3 polypeptide may be determined in any suitable way.

The polypeptide sequence of Rsk-3 is given in Zhao *et al* (1995) *Mol. Cell. Biol.* **15**, 4353-4363 and is also given in the EMBL-GenBank data library under Accession No X85106.

It is preferred if the relative amount of, or intracellular location of, or physical form of the Rsk-3 polypeptide is determined using a molecule which selectively binds to Rsk-3 polypeptide or which selectively binds to a mutant form of Rsk-3 polypeptide. Suitably, the molecule which selectively binds to Rsk-3 or which selectively binds to a mutant of Rsk-3 is an antibody. The antibody may also bind to a natural variant or fragment of Rsk-3 polypeptide.

Antibodies to Rsk-3 are described in Zhao *et al* (1995) *Mol. Cell. Biol.* **15**, 4353-4363 and, in any case, can be made by methods well known in the art. They are also commerically available; for example, anti-Rsk-3 antibody, polyclonal C-20, is available from Santa Cruz Biotechnology Inc, 2161 Delaware Avenue, Santa Cruz, CA 95060, USA. Anti-Rsk-3 C-20 (catalogue number SC-1431) is a goat polyclonal IgG which recognises an epitope corresponding to amino acids 714-733 mapping at the C-terminus of Rsk-3 of human origin. Anti-Rsk-1 and anti-Rsk-2 rabbit polyclonal antibodies are available from Upstate Biotechnology Incorporated, 199 Saranac Avenue, Lake Placid, NY 12946, USA.

It is preferred if the antibodies used are selective for Rsk-3. By "selective for Rsk-3" we mean that they bind Rsk-3 but do not bind substantially to Rsk-1 or Rsk-2 or other polypeptides in the Rsk family. Preferably the antibody binds selectively only to Rsk-3 polypeptide.

Antibodies which can selectively bind to a mutant form of Rsk-3 can be made, for example, by using peptides which encompass the changed amino acid or otherwise modified region of Rsk-3, or by using fusion proteins which express a portion of the Rsk-3 polypeptide which includes the changed amino acid or otherwise modified region. Certain mutations in the Rsk-3 gene are described in the Examples and, in most cases, the change to the amino acid coding region described. In any case, based on the genetic code, it is possible to deduce readily the change in the amino acid sequence. Antibodies which are selective for a mutant Rsk-3 polypeptide as herein disclosed form a further aspect of the invention.

It is preferred if the molecule which selectively binds Rsk-3 or a mutant thereof does not substantially bind the Rsk-1 or Rsk-2 or further members of the Rsk family polypeptides.

Further members of the Rsk family are described, for example, in Deak *et al* (1998) *EMBO J.* 17, 4426-4441, although the Rsk family member is called MSK1.

An alignment of polypeptides related to Rsk-3 is given in Figure 21 from which it can be seen that there are a number of regions of dissimilarity which can be used to design peptides which are selective for Rsk-3. Such peptides may be used to raise Rsk-3-selective antibodies. In particular, the N-terminal region of Rsk-3 is quite distinct from the other polypeptides, and may be used to design Rsk-3-selective peptides and antibodies.

The antibodies may be monoclonal or polyclonal. Suitable monoclonal antibodies may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and applications", J G R Hurrell (CRC Press, 1982), both of which are incorporated herein by reference.

By "the relative amount of Rsk-3 polypeptide" is meant the amount of Rsk-3 polypeptide per unit mass of sample tissue or per unit number of sample cells compared to the amount of Rsk-3 polypeptide per unit mass of known normal tissue or per unit number of normal cells. The relative amount may be determined using any suitable protein quantitation method.

In particular, it is preferred if antibodies are used and that the amount of Rsk-3 is determined using methods which include quantititative western blotting, enzyme-linked immunosorbent assays (ELISA) or quantitative immunohistochemistry.

The neoplastic condition of lesions can also be detected on the basis of the alteration of wildtype Rsk-3 polypeptide. Such alterations can be determined by sequence analysis in accordance with conventional techniques. More preferably, antibodies (polyclonal or monoclonal) are used to detect differences in, or the absence of Rsk-3 polypeptide or peptides derived therefrom. The antibodies may be prepared as discussed herein.

Other techniques for raising and purifying antibodies are well known in the art and any such techniques may be chosen to achieve the preparations claimed in this invention. In a preferred embodiment of the invention, antibodies will immunoprecipitate Rsk-3 proteins from solution as well as react with Rsk-3 protein on Western or immunoblots of polyacrylamide gels. In another preferred embodiment, antibodies will detect Rsk-3 proteins in paraffin or frozen tissue sections, using immunocytochemical techniques.

Preferred embodiments relating to methods for detecting Rsk-3 or its mutations include enzyme linked immunosorbent assays (ELISA), radioimmunoassay (RIA), immunoradiometric assays (IRMA) and immunoenzymatic assays (IEMA), including sandwich assays using monoclonal and/or polyclonal antibodies. Exemplary sandwich assays are

described by David *et al* in US Patent Nos. 4,376,110 and 4,486,530, hereby incorporated by reference.

The intracellular location of Rsk-3 may readily be determined using methods known in the art such as immunocytochemistry in which a labelled antibody (for example, radioactively or fluorescently labelled antibody) is used to bind to Rsk-3 and its location within the cell is determined microscopically. For example, it is possible using this methodology to determine whether the Rsk-3 is located in the cytoplasm or in the nucleus or, if located in both compartments, the proportion of Rsk-3 which is located in each compartment.

In normal cells, such as normal ovary cells, Rsk-3 is located in the cytoplasm as determined by immunocytochemical analysis. In cancer cells an altered distribution of Rsk-3 occurs such that there is a greater proportion of cells in which Rsk-3 is present in the nucleus (as judged by nuclear staining with an appropriate antibody), compared to normal cells.

Methods for detecting altered cellular distribution include immunohistochemistry IHC (for example, where the antibody or a secondary antibody which recognises the first, is labelled with an enzyme, a fluorescent tag, a radioisotope), computer-aided image analysis of IHC stained sections; and flow cytometric analysis of cell nuclei released from fresh tissue or from paraffin sections.

In normal ovarian cells Rsk-3 staining may be found in both the cytoplasm and nucleus, but predominantly in the cytoplasm. The distribution of Rsk-3 in normal cells is cell cycle dependent, showing translocation from the

cytoplasm to the nucleus when cells are stimulated by mitogens to enter the cell cycle. Thus, in a population of normal cells it would be expected that a proportion of the cells would show nuclear staining. In malignant cells, substantially all of the tumour cells would show predominantly nuclear Rsk-3 staining. Contaminating normal cells in a histology section, would of course, show a normal pattern of staining. IHC can be used as an adjunct to routine histology, and may be especially useful, in combination with epithelial or ovarian markers, to detect a low number of metastatic cells.

In post-menopausal women there is unlikely to be a great level of cycling cells and thus of nuclear Rsk-3 staining. In pre-menopausal women there may be more background staining. It may be useful to compare the staining pattern of tissue from the affected ovary and contra-lateral unaffected ovaries.

The relative activity of Rsk-3 can be determined by measuring the activity of the Rsk-3 polypeptide per unit mass of sample tissue or per unit number of sample cells and comparing this activity to the activity of the Rsk-3 polypeptide per unit mass of known normal tissue or per unit number of normal cells. The relative amount may be determined using any suitable enzyme assay. Preferably, the assay is selective for the Rsk-3 polypeptide enzyme activity.

Rsk-3 enzyme activity may be assayed using methods well known in the art. For example, as is described in Zhou *et al* (1995) *Mol. Cell. Biol.* **15**, 4353-4363, the protein kinase activity of Rsk-3 can be assessed with a synthetic peptide substrate (RRRLSSLRA) which corresponds to amino

acids 231 to 239 of the ribosomal S6 protein and is known to be phosphorylated by other RSK isoforms (see Kyriakis, J.M. and Arruch, J., 1994, In J.R. Woodgett (ed), Protein Kinases: frontiers in molecular biology, Oxford University Press, Oxford, incorporated herein by reference). An assay for the protein kinase activity of Rsk-3 is also described in Example 1. S6 peptide substrate for kinase assays can be obtained from Upstate Biotechnology Incorporate, 199 Saranac Avenue, Lake Placid, NY 12946, USA. Other suitable substrates may be used.

Certain mutations in malignant ovarian cancer cell lines appear to render Rsk-3 kinase constitutively active.

Kinase assays may use Rsk-3 from cell lysates, using, for example, immunoprecipitation to obtain Rsk-3 (which may be in a complex with other proteins), or by expressing Rsk3 in $p90^{Rsk}$ null cells, or expression systems. The Rsk-3 could be tagged with a group suitable for purification or immobilisation, or be a fusion protein.

Increased activity relative to normal, quiescent cells, is 2-4 fold for S6 peptide. The relative increase in kinase activity may be substrate dependent.

Altered substrate specificity may be determined by examining a range of known Rsk-3 substrates and demonstrating that the relative efficiency with which they are phosphorylated changes. Alternatively, a protein or peptide library, eg phage display, or combinatorial peptide library, may be screened for molecules which bind to mutant or wildtype Rsk-3 protein.

Phosphorylation of these peptides/proteins can then be examined in a kinase assay.

Activation of Rsk-3 polypeptide can be measured by autophosphorylation, or in kinase assays. Antibodies specific to the autophosphorylated form may be useful for detecting activation.

Rsk-3 has two complete, non-identical, serine/threonine kinase domains. Rsk-3 belongs to the $p90^{Rsk}$ family which has two other members, Rsk-1 and Rsk-2. Although the members of this family are named ribosomal S6 kinases, *in vivo* phosphorylation of the 40S ribosomal subunit which incorporates the S6 protein, is substantially mediated by the distinct p70 family of S6 kinases. The gene encodes a protein of around 83kD and a 6.5kb mRNA transcript. The gene is ubiquitously expressed, however, lung and skeletal muscle have been reported to have particularly high levels of expression (Zhao *et al* (1995) *Mol Cell Biol* **15**, 4345-4363).

The kinase activity of Rsk-3 can be demonstrated *in vitro* by incubating Rsk-3 (eg isolated from cell lysates by immunoprecipitation with an Rsk-3 specific antibody, or using an epitope or His tagged recombinant Rsk-3), with [γ-^{32}P]ATP and a suitable substrate. Rsk-3 isolated from quiescent cells has a basal level of kinase activity, however this can be increased by prior treatment of the cells with various stimuli. Agents which have been shown to produce a 2-6 fold increase in Rsk-3 activity in such assays (using Rsk-3 expressed in COS cells) include serum, insulin, EGF, PDGF, IGF, PMA (phorbol 12-myristate 13-acetate), and TPA (12-O-tetradecanoylphorbol-13-acetate). Serum stimulation of COS cells produces a peak in Rsk-3 kinase activity at 20 minutes.

A putative consensus sequence for RSK protein substrates is R-X-X-S. Rsk-3 expressed in COS cells has been reported to phosphorylate the following substrates *in vitro* (Zhao *et al* (1995) *Mol Cell Biol* **15**, 4345-4363):

Substrate	Relative efficiency
S6 peptide (amino acids 231-239)	100
Kemptide	122
histone H1	8
histone H2A	7
histone H2B	55
histone H3	5
histone H4	3
recombinant protein phosphatase 1 G subunit (RG1);	11
c-Fos	10
c-Jun	2
S40 ribosomal subunit	12
casein	10

In vitro kinase assays can also be used to measure the effect of putative activators and deactivators of Rsk-3 on Rsk-3 kinase activity. For example, addition of protein phosphatase 2A decreases Rsk-3 kinase activity, to a level below the basal level of activity seen for Rsk-3 from quiescent cells (Zhao *et al* (1995) *Mol Cell Biol* **15**, 4345-4363).

A similar system can be used to detect phosphorylation of Rsk-3 *in vitro*. Autophosphorylation, phosphorylation by added kinases, or de-phosphorylation by added phosphatases can be detected. For example, inclusion of ERK2 results in increased phosphorylation of Rsk-3, however, this does not result in an increase in Rsk-3 kinase activity (Zhao *et al* (1995) *Mol Cell Biol* **15**, 4345-4363).

The $p90^{Rsk}$ family, including Rsk-3 lie on the MAP kinase intracellular signalling pathway. Growth factors and hormone which activate Rsk-3 may do so via MEK-1, a kinase on this pathway. Inhibitors of MEK-1 and transfection with dominant negative forms of MEK-1 can reduce EGF stimulation of Rsk-3 kinase activation, measured as described above. Transfection of cells with active MEK-1 is also sufficient to activate Rsk-3 kinase activity. The kinases ERK1 and ERK2, which are substrates for MEK1, both co-immunoprecipitate from cell extracts with Rsk-3. Deletion mutants of Rsk-3 have shown that the C-terminal 33 amino acids of Rsk-3 are required for this binding. Although incubation of Rsk-3 with ERK2 has been shown to result in phosphorylation of Rsk-3, this is not sufficient to activate Rsk-3 in kinase assays. Nor does phosphorylation of ERK1 and ERK2 by incubation with MEK1 result in Rsk-3 kinase activation (Zhao *et al* (1995) *J. Biol. Chem.* **271**, 29773-29779). Insulin has been shown to activate Rsk-3 in skeletal muscle, and thus glycogen synthase, via the MAP kinase pathway component JNK (Moxham *et al* (1996) *J. Biol. Chem.* **271**, 30765-30773).

Phosphorylation of the known Rsk-3 substrates is mediated by the N-terminal kinase domain. Site directed mutagenesis experiments for Rsk-3 have shown that mutation of the residues K91 and K444 in the ATP-

binding sites of the N-terminal and C-terminal kinase domains respectively, can abolish the kinase activity of the respective domains. Both kinase domains are required for maximal activity (including autophosphorylation), however the presence of even an inactive C-terminal domain may modulate the activity of the N-terminal domain (Bjorbeak *et al* (1995) *J. Biol. Chem.* **270**, 18848-18852). Activation of the $p90^{Rsk}$ family is mediated, at least in part, by phosphorylation of serine and threonine residues by other kinases and by autophosphorylation. Autophosphorylation of Rsk-3 is seen in response to agents that activate its kinase activity, and is a marker of activation. De-phosphorylation of Rsk-3 by protein phosphatase 2A deactivates Rsk-3 kinase activity. Residues in Rsk-3 indicated as potential sites for regulatory phosphorylation (by MAP kinases) by analogy to Rsk-1 and Rsk-2 (ie S218 and T570) are not required for basal Rsk-3 kinase activity or activation by EGF, as demonstrated by site-directed mutation experiment (Bjorbeak *et al* (1995) *J. Biol. Chem.* **270**, 18848-18852). Nor does replacement of these residues with negatively charged residues to mimic phosphorylation result in constitutive activation.

Rsk-3 in quiescent cells is mostly detected in the cytoplasm when investigated using immunohistochemistry. Stimulation of cells resulting in entry to the cell cycle, results in movement of Rsk-3 into the nucleus, within 1 hour of stimulation.

Rsk-3 shows 75% and 84% amino acid homology to Rsk-1 and Rsk-2 respectively. Rsk-3 also has a stretch of 33 amino acids at the N-terminal which are not found in Rsk-1 or Rsk-2. This region of the Rsk-3 protein is particularly useful for raising antibodies specific to Rsk-3, for example

a suitable antigen is the peptide KFAVRRFFSVYLRR. Divergent sequences in the C-terminal region of the $p90^{Rsk}$s may also be suitable for raising antibodies specific to individual family members. The three family members show differences in tissue distribution, have different *in vitro* substrate preferences, and show differential activation by various kinases and phosphatases. Rsk-1 is predominantly expressed in haemopoietic cells, liver and intestine, whereas Rsk-2 has more widespread expression. Both Rsk-1 and Rsk-2 are phosphorylated and partially activated by ERK2 *in vitro*, and are activated by a MEK-1 pathway. Much of the published data on the $p90^{Rsk}$s fails to distinguish between the family members due to lack of specific antibodies when the data was generated. Mutations to Rsk-2 are responsible for the inherited syndrome Coffin-Lowry, which has a phenotype characterised by pyschomotor retardation. The mutated Rsk-2 allele identified encoded proteins which were truncated or inactive in an S6 peptide kinase assay (Trivier *et al* (1997) *Nature* 384, 567-570). Both Rsk-1 and 2 also have putative nuclear localisation signals, distinct from the putative N-terminal nuclear localisation signal identified in Rsk-3 (Zhao *et al* (1995) *Mol Cell Biol* 15, 4345-4363).

Although the MAP kinase pathway is known to mediate mitogenic signals, it has divergent effects and also stimulates cell responses which are not causal for cell division. Thus it is not obvious that any given kinase or target in the pathway might exert a carcinogenic effect if its function were modulated. Similarly it is not obvious that a given component of the pathway, especially one several steps removed from the receptor would be a suitable target for therapeutic intervention. Indeed, components closer to the receptor eg MEK-1, and thus more likely to have a more general effect would appear to be more obvious targets. The data suggesting that

Rsk-3 mediates the effect of insulin on glycogen synthesis, and the preferential expression of Rsk-3 in skeletal muscle, are not suggestive of a role for Rsk-3 in carcinogenesis.

The invention also provides an antibody which reacts with a mutant Rsk-3 polypeptide or fragment thereof, wherein said mutant Rsk-3 is a mutant found in a cancer cell. Preferably, the antibody does not react with wild-type Rsk-3 polypeptide or Rsk-1 polypeptide or Rsk-2 polypeptide. Such antibodies are useful in the diagnostic assays and methods of the invention and may be made, for example, by using peptides whose sequence is derived from mutant Rsk-3 polypeptide as immunogens. Mutant Rsk-3 polypeptides are disclosed in the Examples.

The invention also provides a nucleic acid which selectively hybridises to a nucleic acid encoding a mutant Rsk-3 polypeptide, which mutant is one found in a cancer cell. Such nucleic acids are useful in the diagnostic assays and methods of the invention.

Whilst any hypothesis on the involvement of Rsk-3 in cancer is not binding, mutations to the Rsk-3 gene may result in a shift in function incorporating both loss and gain of function features. Carcinogenesis may result from the altered balance between wild-type and mutant functions, which may also be affected by the loss of the second allele. As will be clear from the disclosure above, the events which may be clinically relevant to detect include detections in the coding sequence of the first allele, loss by deletion or inactivation of the second allele, and altered behaviour of the gene product.

It will be appreciated that in respect of the certain nucleic acid-based methods of diagnosis, determination of susceptibility and prediction of relative prospects of outcome, the methods involve determining whether the status of Rsk-3 nucleic acid (whether DNA or mRNA) is altered in a sample being tested compared to a sample from an equivalent tissue or other source which is known to be normal or disease free.

As is discussed in more detail below, therapeutically, both restoration of wild-type function (for example, by gene therapy), or inhibition of the mutant function (for example, using kinase inhibitors, may be useful to reverse the effect of the mutation).

Peptides based on the mutant sequences may be useful in stimulating an immune response.

A further aspect of the invention provides a method of treating cancer comprising the step of administering to the patient a nucleic acid which selectively hybridises to the Rsk-3 gene, or a nucleic acid which hybridises selectively to Rsk-3 cDNA.

A further aspect of the invention provides a method of treating cancer comprising the step of administering to the patient a nucleic acid which encodes the Rsk-3 polypeptide or a functional variant or portion or fusion thereof.

The invention also includes the administration of all or part of the Rsk-3 gene or cDNA to a patient with a cancer.

Suitably, the nucleic acid which is administered to the patient is a nucleic acid which encodes the Rsk-3 polypeptide or a functional variant or portion thereof. Preferably, the Rsk-3 polypeptide is a wild-type polypeptide or a variant polypeptide which has substantially wild-type activities. It is less preferred if the Rsk-3 polypeptide is a polypeptide with mutations which are found in cancer cells such as ovarian cancer cells; however, as discussed below, such polypeptides may be useful in provoking an anti-cancer cell immune response. Thus, according to the present invention, a method is also provided of supplying wild-type Rsk-3 function to a cell which carries mutant Rsk-3 alleles. Supplying such a function should suppress neoplastic growth of the recipient cells. The wild-type Rsk-3 gene or a part of the gene may be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation, the gene will be expressed by the cell from the extrachromosomal location. If a gene fragment is introduced and expressed in a cell carrying a mutant Rsk-3 allele, the gene fragment should encode a part of the Rsk-3 protein which is required for non-neoplastic growth of the cell. More preferred is the situation where the wild-type Rsk-3 gene or a part thereof is introduced into the mutant cell in such a way that it recombines with the endogenous mutant Rsk-3 gene present in the cell. Such recombination requires a double recombination event which results in the correction of the Rsk-3 gene mutation. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art, and any suitable vector may be used. Methods for introducing DNA into cells such as electroporation, calcium phosphate co-precipitation and viral transduction are known in the art, and the choice of method is within the competence of the routineer. Cells transformed with the wild-type Rsk-3 gene can be

used as model systems to study cancer remission and drug treatments which promote such remission.

As generally discussed above, the Rsk-3 gene or fragment, where applicable, may be employed in gene therapy methods in order to increase the amount of the expression products of such genes in cancer cells. Such gene therapy is particularly appropriate for use in both cancerous and pre-cancerous cells, in which the level of Rsk-3 polypeptide is absent or diminished or otherwise changed compared to normal cells. It may also be useful to increase the level of expression of a given Rsk-3 gene even in those tumour cells in which the mutant gene is expressed at a "normal" level, but the gene product is not fully functional or has an altered function.

Gene therapy would be carried out according to generally accepted methods, for example, as described by Friedman, 1991. Cells from a patient's tumour would be first analyzed by the diagnostic methods described herein, to ascertain the production of Rsk-3 polypeptide and its physical form (ie what mutations it contains) in the tumour cells. A virus or plasmid vector (see further details below), containing a copy of the Rsk-3 gene linked to expression control elements and capable of replicating inside the tumour cells, is prepared. Suitable vectors are known, such as disclosed in US Patent 5,252,479 and PCT published application WO 93/07282. The vector is then injected into the patient, either locally at the site of the tumour or systemically (in order to reach any tumour cells that may have metastasized to other sites). If the transfected gene is not permanently incorporated into the genome of each

of the targeted tumour cells, the treatment may have to be repeated periodically.

Gene transfer systems known in the art may be useful in the practice of the gene therapy methods of the present invention. These include viral and nonviral transfer methods. A number of viruses have been used as gene transfer vectors, including papovaviruses, eg SV40 (Madzak *et al*, 1992), adenovirus (Berkner, 1992; Berkner *et al*, 1988; Gorziglia and Kapikian, 1992; Quantin *et al*, 1992; Rosenfeld *et al*, 1992; Wilkinson *et al*, 1992; Stratford-Perricaudet *et al*, 1990), vaccinia virus (Moss, 1992), adeno-associated virus (Muzyczka, 1992; Ohi *et al*, 1990), herpesviruses including HSV and EBV (Margolskee, 1992; Johnson *et al*, 1992; Fink *et al*, 1992; Breakfield and Geller, 1987; Freese *et al*, 1990), and retroviruses of avian (Brandyopadhyay and Temin, 1984; Petropoulos *et al*., 1992), murine (Miller, 1992; Miller *et al*, 1985; Sorge *et al*, 1984; Mann and Baltimore, 1985; Miller *et al*, 1988), and human origin (Shimada *et al*, 1991; Helseth *et al*, 1990; Page *et al*, 1990; Buchschacher and Panganiban, 1992). To date most human gene therapy protocols have been based on disabled murine retroviruses.

Nonviral gene transfer methods known in the art include chemical techniques such as calcium phosphate coprecipitation (Graham and van der Eb, 1973; Pellicer *et al*, 1980); mechanical techniques, for example microinjection (Anderson *et al*, 1980; Gordon *et al*, 1980; Brinster *et al*, 1981; Constantini and Lacy, 1981); membrane fusion-mediated transfer *via* liposomes (Felgner *et al*, 1987; Wang and Huang, 1989; Kaneda *et al*, 1989; Stewart *et al*, 1992; Nabel *et al*, 1990; Lim *et al*, 1992); and direct DNA uptake and receptor-mediated DNA transfer (Wolff *et al*, 1990; Wu

et al, 1991; Zenke *et al,* 1990; Wu *et al,* 1989b; Wolff *et al,* 1991; Wagner *et al,* 1990; Wagner *et al,* 1991; Cotten *et al,* 1990; Curiel *et al,* 1991a; Curiel *et al,* 1991b). Viral-mediated gene transfer can be combined with direct *in vivo* gene transfer using liposome delivery, allowing one to direct the viral vectors to the tumour cells and not into the surrounding nondividing cells. Alternatively, the retroviral vector producer cell line can be injected into tumours (Culver *et al,* 1992). Injection of producer cells would then provide a continuous source of vector particles. This technique has been approved for use in humans with inoperable brain tumours.

Other suitable systems include the retroviral-adenoviral hybrid system described by Feng *et al* (1997) *Nature Biotechnology* **15**, 866-870, or viral systems with targeting ligands such as suitable single chain Fv fragments.

In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein, and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization, and degradation of the endosome before the coupled DNA is damaged.

Liposome/DNA complexes have been shown to be capable of mediating direct *in vivo* gene transfer. While in standard liposome preparations the gene transfer process is nonspecific, localized *in vivo* uptake and expression have been reported in tumour deposits, for example, following direct *in situ* administration (Nabel, 1992).

Gene transfer techniques which target DNA directly to ovarian and breast tissues, eg epithelial cells of the breast or ovaries, is preferred. Receptor-mediated gene transfer, for example, is accomplished by the conjugation of DNA (usually in the form of covalently closed supercoiled plasmid) to a protein ligand *via* polylysine. Ligands are chosen on the basis of the presence of the corresponding ligand receptors on the cell surface of the target cell/tissue type. One appropriate receptor/ligand pair for introduction of the therapeutic gene into breast tumour cells may include the estrogen receptor and its ligand, estrogen (and estrogen analogues). These ligand-DNA conjugates can be injected directly into the blood if desired and are directed to the target tissue where receptor binding and internalization of the DNA-protein complex occurs. To overcome the problem of intracellular destruction of DNA, coinfection with adenovirus can be included to disrupt endosome function.

In the case where replacement gene therapy using a functionally wild-type Rsk-3 is used, it may be useful to monitor the treatment by detecting the presence of Rsk-3 mRNA or polypeptide, or functional Rsk-3, at various sites in the body, including the targeted tumour, sites of metastasis, blood serum, and bodily secretions/excretions, for example urine.

A further aspect of the invention provides a method of treating cancer comprising the step of administering to the patient an effective amount of Rsk-3 polypeptide or a fragment or variant or fusion thereof to ameliorate the cancer.

Peptides which have Rsk-3 activity can be supplied to cells which carry mutant or missing Rsk-3 alleles. The sequence of the Rsk-3 protein is disclosed in the Figures. Protein can be produced by expression of the cDNA sequence in bacteria, for example, using known expression vectors. Alternatively, Rsk-3 polypeptide can be extracted from Rsk-3 -producing mammalian cells. In addition, the techniques of synthetic chemistry can be employed to synthesize Rsk-3 protein. Any of such techniques can provide the preparation of the present invention which comprises the Rsk-3 protein. The preparation is substantially free of other human proteins. This is most readily accomplished by synthesis in a microorganism or *in vitro*.

The Rsk-3 gene or cDNA can be expressed by any suitable method. Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps)

A variety of methods have been developed to operably link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini

with their 3′-5′-exonucleolytic activities, and fill in recessed 3′-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

A further aspect of the invention provides a method of treating cancer, the method comprising administering to the patient an effective amount of a mutant Rsk-3 polypeptide or fragment thereof, or an effective amount of a nucleic acid encoding a mutant Rsk-3 polypeptide or fragment thereof, wherein the said mutant Rsk-3 is a mutant found in a cancer cell and the amount of said mutant polypeptide or amount of said nucleic acid is effective to provoke an anti-cancer cell immune response in said patient.

The mutant peptide or peptide-encoding nucleic acid constitutes a tumour or cancer vaccine. It may be administered directly into the patient, into the affected organ or systemically, or applied *ex vivo* to cells derived from the patient or a human cell line which are subsequently administered to the patient, or used *in vitro* to select a subpopulation from immune cells derived from the patient, which are then re-administered to the patient. If the nucleic acid is administered to cells *in vitro*, it may be useful for the

cells to be transfected so as to co-express immune-stimulating cytokines, such as interleukin-2. The mutant Rsk-3 polypeptide or peptide fragment therefore comprising the mutation may be substantially pure, of combined with an immune-stimulating adjuvant, or used in combination with immune-stimulatory cytokines, or be administered with a suitable delivery system, for example liposomes. The peptide may also be tagged, or be a fusion protein. The nucleic acid may be substantially pure, or contained in a suitable vector or delivery system. The peptide or peptide encoded by the nucleic acid may be a fusion protein, for example with β2-microglobulin.

It is particularly useful if the cancer vaccine is administered in a manner which produces a cellular immune response, resulting in cytoxic tumour cell killing by NK cells or cytotoxic T cells (CTLs). Strategies of administration which activate T helper cells are particularly useful. It may also be useful to stimulate a humoral response. It may be useful to co-adminster certain cytokines to promote such a response, for example interleukin-2, interleukin-12, interleukin-6, or interleukin-10. It may also be useful to target the vaccine to specific cell populations, for example antigen presenting cells, either by the site of injection, use of targeting vectors and delivery systems, or selective purification of such a cell population from the patient and *ex vivo* administration of the peptide or nucleic acid (for example dendritic cells may be sorted as described in Zhou *et al* (1995) *Blood* **86**, 3295-3301; Roth *et al* (1996) *Scand. J. Immunology* **43**, 646-651).

Patients to whom the therapy is to be given, may have their tumours typed for mutation so that the appropriate mutant peptide or nucleic acid can be used in the method or vaccine of the invention.

A further aspect of the invention therefore provides a vaccine effective against cancer or cancer or tumour cells comprising an effective amount of a mutant Rsk-3 polypeptide or mutant-containing fragment thereof, or comprising a nucleic acid encoding such a polypeptide or mutant-containing fragment thereof.

Figure 18 shows the wild-type sequences for exons 2, 3, 8 and 14 of Rsk-3 and the sequences of mutants from various tumours or tumour cell lines in these exons. This information can readily be used to design suitable peptides and nucleic acids for use in the methods and vaccines of the invention using methods well known in the art.

A still further aspect of the invention provides a method of treating cancer comprising the step of administering to the patient an effective amount of a compound which inhibits Rsk-3 polypeptide function, or the function of a mutant Rsk-3 polypeptide found in a tumour cell.

Suitable compounds for use in this method of the invention include antibodies or fragments or variants thereof which inhibit Rsk-3 activity, or antisense molecules which inhibit the expression of Rsk-3. It is also preferred if the inhibitors are ones which are selective for a mutant form of Rsk-3 present in a cancer cell. Preferably, the inhibitors do not substantially inhibit Rsk-1 or Rsk-2.

Alternatively, suitable compounds may be obtained by screening.

Screening compounds by using the Rsk-3 polypeptide or binding fragment thereof in any of a variety of drug screening techniques may be used.

The Rsk-3 polypeptide or fragment employed in such a test may either be free in solution, affixed to a solid support, or borne on a cell surface. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant polynucleotides expressing the polypeptide or fragment, preferably in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays. One may measure, for example, for the formation of complexes between a Rsk-3 polypeptide or fragment and the agent being tested, or examine the degree to which the formation of a complex between a Rsk-3 polypeptide, or fragment and a known ligand is interfered with by the agent being tested.

Thus, the present invention provides methods of screening for drugs comprising contacting such an agent with a Rsk-3 polypeptide or fragment thereof and assaying (i) for the presence of a complex between the agent and the Rsk-3 polypeptide or fragment, or (ii) for the presence of a complex between the Rsk-3 polypeptide or fragment and a ligand, by methods well known in the art. In such competitive binding assays the Rsk-3 polypeptide or fragment is typically labeled. Free Rsk-3 polypeptide or fragment is separated from that present in a protein:protein complex and the amount of free (ie uncomplexed) label is a measure of the binding of the agent being tested to Rsk-3 or its interference with Rsk-3:ligand binding, respectively.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the Rsk-3 polypeptides and is described in detail in Geysen, PCT published application WO 84/03564, published on September 13, 1984. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with Rsk-3 polypeptide and washed. Bound Rsk-3 polypeptide is then detected by methods well known in the art.

Purified Rsk-3 can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to the polypeptide can be used to capture antibodies to immobilize the Rsk-3 polypeptide on the solid phase.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of specifically binding the Rsk-3 polypeptide compete with a test compound for binding to the Rsk-3 polypeptide or fragments thereof. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants of the Rsk-3 polypeptide.

A further technique for drug screening involves the use of host eukaryotic cell lines or cells (such as described above) which have a mutant Rsk-3 gene. These host cell lines or cells are defective at the Rsk-3 polypeptide level. The host cell lines or cells are grown in the presence of drug compound. The rate of growth of the host cells is measured to determine

if the compound is capable of regulating the growth of Rsk-3 defective cells.

Additionally or alternatively, rational drug design may be used. The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact (eg agonists, antagonists, inhibitors) in order to fashion drugs which are, for example, more active or stable forms of the polypeptide, or which, eg enhance or interfere with the function of a polypeptide *in vivo*. See, eg Hodgson, 1991. In one approach, one first determines the three-dimensional structure of a protein of interest (eg Rsk-3 polypeptide) or, for example, of the Rsk-3-receptor or ligand complex, by x-ray crystallography, by computer modeling or most typically, by a combination of approaches. Less often, useful information regarding the structure of a polypeptide may be gained by modeling based on the structure of homologous proteins. An example of rational drug design is the development of HIV protease inhibitors (Erickson *et al*, 1990). In addition, peptides (eg Rsk-3 polypeptide) are analyzed by an alanine scan (Wells, 1991). In this technique, an amino acid residue is replaced by Ala, and its effect on the peptide's activity is determined. Each of the amino acid residues of the peptide is analyzed in this manner to determine the important regions of the peptide.

It is also possible to isolate a target-specific antibody, selected by a functional assay, and then to solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional,

pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analog of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically or biologically produced banks of peptides. Selected peptides would then act as the pharmacore.

Thus, one may design drugs which have, for example, improved Rsk-3 polypeptide activity or stability or which act as inhibitors, agonists, antagonists, etc of Rsk-3 polypeptide activity. By virtue of the availability of cloned Rsk-3 sequences, sufficient amounts of the Rsk-3 polypeptide may be made available to perform such analytical studies as x-ray crystallography. In addition, the knowledge of the Rsk-3 protein sequence provided herein will guide those employing computer modeling techniques in place of, or in addition to x-ray crystallography.

Cells and animals which carry a mutant Rsk-3 allele can be used as model systems to study and test for substances which have potential as therapeutic agents. The cells are typically cultured epithelial cells. These may be isolated from individuals with Rsk-3 mutations, either somatic or germline. Alternatively, the cell line can be engineered to carry the mutation in the Rsk-3 allele, using methods well known in the art. After a test substance is applied to the cells, the neoplastically transformed phenotype of the cell is determined. Any trait of neoplastically transformed cells can be assessed, including anchorage-independent growth, tumourigenicity in nude mice, invasiveness of cells, and growth factor dependence. Assays for each of these traits are known in the art.

Animals for testing therapeutic agents can be selected after mutagenesis of whole animals or after treatment of germline cells or zygotes. Such treatments include insertion of mutant Rsk-3 alleles, usually from a second animal species, as well as insertion of disrupted homologous genes. Alternatively, the endogenous Rsk-3 gene(s) of the animals may be disrupted by insertion or deletion mutation or other genetic alterations using conventional techniques (Capecchi, 1989; Valancius and Smithies, 1991; Hasty *et al,* 1991; Shinkai *et al,* 1992; Mombaerts *et al,* 1992; Philpott *et al,* 1992; Snouwaert *et al,* 1992; Donehower *et al,* 1992). After test substances have been administered to the animals, the growth of tumours must be assessed. If the test substance prevents or suppresses the growth of tumours, then the test substance is a candidate therapeutic agent for the treatment of the cancers identified herein. These animal models provide an extremely important testing vehicle for potential therapeutic products.

Active Rsk-3 molecules can be introduced into cells by microinjection or by use of liposomes, for example. Alternatively, some active molecules may be taken up by cells, actively or by diffusion. Extracellular application of the Rsk-3 gene product may be sufficient to affect tumour growth. Supply of molecules with Rsk-3 activity should lead to partial reversal of the neoplastic state. Other molecules with Rsk-3 activity (for example, peptides, drugs or organic compounds) may also be used to effect such a reversal. Modified polypeptides having substantially similar function are also used for peptide therapy.

Further aspects of the invention provide a pharmaceutical composition comprising a gene therapy vector including a nucleic acid which encodes

the Rsk-3 polypeptide or a functional variant or portion or fusion thereof and pharmaceutically acceptable carrier; a pharmaceutical composition comprising a gene therapy vector including a nucleic acid which selectively hybridises to the Rsk-3 gene, or a mutant allele thereof, or a Rsk-3 cDNA, or a mutant allele thereof, and a pharmaceutically acceptable carrier; a pharmaceutical composition comprising Rsk-3 polypeptide or a fragment or variant or fusion thereof, and a pharmaceutically acceptable carrier.

Suitable gene therapy vectors are described above. Suitable Rsk-3 polypeptides are described above.

By "pharmaceutically acceptable" is included that the formulation is sterile and pyrogen free. Suitable pharmaceutical carriers are well known in the art of pharmacy.

The present invention will now be described in more detail with reference to the following Examples and Figures wherein

Fig 1. Genetic and physical map. A schematic genetic map comprising of the Genethon map and the integrated map around 6q26-27 (Cooke *et al*, 1996) The STS content is shown on each PAC. PACs 427A4, 168L15 and 497J21 which were sequenced (size in parenthesis, Acc. No. Z98049, AL022069, AL023775 respectively) are shown in bold and the p90 Rsk-3 cDNA is shown underneath. All the markers are prefixed by D6S except 17631 and 11162 which are prefixed by stSG. 'A' refers to the revised minimal region of allele loss based on data reported previously (Cooke *et al*, 1996). 'B' refers to the published sequence (Acc. No. AF019664).

Genetic distances are in centimorgans. The two WI markers shown are ESTs. Distance between D6S193 and p90 Rsk-3 is 142 kb, and between D6S297 and D6S193 is 55 kb. The gene for p90 Rsk-3 is the only gene identified in the region between D6S297 and D6S264 of which 537 Kb has been sequenced from D6S297 till the end of PAC 168L15.

Fig. 2. Autoradiograph of SSCP gels for exon 8 (A) and exon 14 (B) of malignant ovarian tumours. The tumour samples (T) are identified on the top of each lane and arrows point to the abnormal bands. N= normal DNA from peripheral blood of each individual patient as control. The dried gels were exposed overnight with intensifying screens at -70°C. (C) Alignment of amino acid residues of mouse 1 and 2 (P18654 and P186543), rat (A53300), chicken (P18652), xenopus(P10665) p90 Rsk and human p90 Rsk-1 (L07597), Rsk-2 (L07599) and Rsk-3 (X85106) was performed by the Pileup program of GCG and displayed using BoxShade 3.2. The exons 2, 3, 8 and 14 of p90 Rsk-3 are underlined. The glycine rich loop of each catalytic domain is indicated by an asterisk. Mutated amino acid residues in each exon are indicated by a tick (✓) under the residue. The alignment is shown upto codon 440 for p90 Rsk-3 encompassing exons 1-14.

Fig. 3 (A) Chromatogram of sequence from nucleotides 1486-1506 in exon 14 of N58&T58. (B) Chromatogram of sequence from nucleotides 838-867 in exon 8 of N37&T37. The mutation at 855 is a polymorphism and differs from the sequence of Rsk-3. (C) Chromatogram of sequence from nucleotide 289-315 in exon 2 of cell line OAW42 with normal placental DNA as control. The sequence of the forward strand is shown graphically (top and bottom) with the text above in both panels. The

amino-acid substitution from the normal coding sequence of Rsk-3 is indicated below the site of mutation (arrow).

Fig. 4 (A) Western blot showing expression of p90 Rsk-3 in ovarian cancer cell lines identified on the top of each lane. The blot was exposed for 5 minutes after adding the detection reagent (ECL Plus, Amersham). Markers (K) are shown on the right of the panel. (B) Western blot showing Erk-1 and Erk-2 in ovarian cancer cell lines. The cells were stimulated for 15 minutes with EGF (+/-) after having been serum starved for 6 hours (0.1% foetal calf serum) at a concentration of 100ng/ml. The blot was exposed for 3 minutes and the specific Erk1 (p44)and Erk2 (p42) proteins are indicated by an arrow. (C) Western blot showing p90 Rsk-3 (arrow) in ovarian cancer cell lines. The cells were stimulated for 15 minutes with 100 ng/ml EGF (+/-) after having been serum starved for 6 hours (0.1% foetal calf serum).

Fig 5 (A) *In vitro* immune complex kinase assay of p90 Rsk-3 in ovarian cancer cell lines before (-) and after (+) stimulation with EGF at 100ng/ml for 15 minutes following serum starvation for 6 hours. Arrow indicates the phosphorylated p90 Rsk-3 protein. The image was obtained after overnight exposure using Phosphor Imager. (B) The graph represents the mean of results from two experiments with standard error bars. The absolute count shown on the Y axis represents ^{32}P incorporated into p90 Rsk-3 as determined by Phosphor Imager analysis after exposure for 12 hours. (C) The results of phosphorylation of substrate S6 peptide by immunoprecipitated p90 Rsk-3 in ovarian cancer cell lines and HeLa. The mean absolute count using IgG as control was 459.27. (D) The results of phosphorylation of substrate S6 peptide by immunoprecipitated

p90 Rsk-2 in six ovarian cancer lines. The mean absolute count using IgG as a control was 515.66. For (C) and (D) the mean absolute count (logarithmic scale) from three experiments was obtained from Phosphor Imager analysis (after subtraction of background using IgG as control) after a 2 hour exposure and is shown with standard error bars.

Figure 6 shows the nucleotide sequence (double stranded) of a cDNA encoding human p90 and the ribosomal S6 kinase-3 (Rsk-3) encoded polypeptide; intron-exon boundaries are marked by vertical arrows and the exons are numbered. The Rsk-3 cDNA sequence is also given in Zhao *et al* (1995) *Mol. Cell. Biol.* **15**, 4353-4363, incorporated herein by reference, and this sequence is publicly available from the EMBL-GenBank data libary under Accession No X85106.

Figure 7 shows exon 1 and flanking intron sequences of the Rsk-3 gene. The exon boundary is marked with vertical lines and the word "exon". The sequences of primers useful in amplifying exon 1 are given.

Figure 8 shows exon 2 and flanking intron sequences of the Rsk-3 gene. The exon boundary is marked with vertical lines and the word "exon". The sequences of primers useful for amplifying exon 2 are given.

Figure 9 shows exon 3 and flanking intron sequences of the Rsk-3 gene. The exon boundary is marked with vertical lines and the word "exon". The sequences of primers useful in amplifying exon 3 are given.

Figure 10 shows exon 4 and flanking intron sequences of the Rsk-3 gene. The exon boundary is marked with vertical lines and the word "exon".

The sequences of primers useful in amplifying exon 4 are marked by horizontal arrows.

Figure 11 shows exon 5 and flanking intron sequences of the Rsk-3 gene. The exon boundary is marked with vertical lines and the word "exon". The sequences of primers useful in amplifying exon 5 are marked with horizontal arrows.

Figure 12 shows exon 6 and flanking intron sequences of the Rsk-3 gene. The exon boundary is marked with vertical lines and the word "exon". The sequences of primers useful in amplifying exon 6 are given.

Figure 13 shows the sequence of primers which may be used to amplify the exons of the Rsk-3 gene. Lower case letters indicate genomic DNA sequence, and upper case letters indicate cDNA sequence. The cDNA sequence numbers are those according to the Rsk-3 nucleotide sequence given in the EMBL-GenBank data library under Accession No X85106.

Figure 14 shows the nucleotide sequence and encoded amino acid sequence of the human Rsk-2 cDNA.

Figure 15 shows the nucleotide sequence and encoded amino acid sequence of the human Rsk-1 cDNA.

Figure 16(a) shows part of the Gen Bank database entry for the human sequence of PAC clone 427A4 which contains part of the Rsk-3 gene.

Figure 16(b) shows part of the Gen Bank database entry for the human sequence of PAC clone 168L15 which contains part of the Rsk-3 gene.

Figure 16(c) shows part of the Gen Bank database entry for the human sequence of PAC clone 497J21.

The relationship between these three PAC clones is given in Figure 1. The human sequence of each PAC clone is publicly available from, for example, Gen Bank by accessing the appropriate World Wide Web site. The human sequence of the PAC clones can be aligned into a contiguous sequence which contains the entire Rsk-3 gene. The feature tables in Figures 16(a), (b) and (c) indicate the position of parts of the Rsk-3 gene. The exon-intron structure in the contiguous genomic gene is readily determined by reference to the Rsk-3 cDNA sequence in Figure 6.

Figure 17 is the nucleotide sequence and encoded amino acid sequence of the human Rsk-3 cDNA as in the EMBL-Gen Bank data library under Accession No X85106.

Figure 18 shows the wild-type sequences for exons 2, 3, 8 and 14 of Rsk-3 and the sequences of mutations from various tumours or tumour cell lines in these exons.

Figure 19 shows immunohistochemistry data for Rsk-3 staining pattern in ovarian tumours.

Figure 20 shows the result of measuring Rsk-3 activity in breast cancer cell lines.

Figure 21 shows an amino acid sequence alignment for polypeptides related to Rsk-3. Peptides which are specific for Rsk-3 can be readily identified, particularly in the N-terminal region of Rsk-3. Such polypeptides may be used to make Rsk-3-selective antibodies.

Example 1: Identification of the p90 ribosomal S6 kinase-3 gene on chromosome 6q27 as the gene mutated in sporadic epithelial ovarian cancer

Summary

In sporadic epithelial ovarian cancer karyotypic and allele loss studies have identified several chromosomal regions including chromosome 6q as containing genes that might contribute to the pathogenesis[1]. A high frequency of allele loss at D6S193 (62%) on chromosomal arm 6q27 in malignant ovarian tumours has been shown previously and mapped the minimal region of allele loss between D6S297 and D6S264 (3cM)[2,3]. The gene for p90 ribosomal S6 kinase-3 (p90 Rsk-3) was identified by mapping and sequencing to be within 50 kB of D6S193. The p90 Rsk family of serine-threonine kinases is unusual in having two catalytic domains and have been shown to be signalling intermediates downstream of ERK in the RAS-RAF-MEK-ERK signalling pathway[4-8]. In this work significant missense mutations have been identified in the catalytic domains of the gene in 50% of malignant and borderline sporadic ovarian tumours and in ovarian cancer cell lines. Constitutive activation of catalytic activity of p90 Rsk-3 in four ovarian cancer cell lines with

mutations is shown. These results suggest that mutations in p90 Rsk-3 gene may represent one of the earliest events in the development of epithelial ovarian cancer.

An integrated genetic map of chromosome 6q24-27 has been constructed previously using the Genethon map as a backbone[3] (Fig. 1). One non-chimaeric YAC 171A12 (ICI YAC library) was identified which was shown to contain D6S193, D6S297, D6S1585 and D6S133. Simultaneously, PACs were isolated from a PAC library[19] for all the markers in this region and were ordered into a contig by STS content and fingerprinting[20]. Two PACs, 119M17 and 427A4, were selected for sequencing and the latter has already been completely sequenced (EMBL Acc. No Z98049). The orientation of the minimal overlapping set of PACs in relation to the genetic map is shown in Fig. 1. The PAC 427A4 contained STSs stSG11162, stSG17631 which are not present in the YAC 171A12. This suggested that there was an internal deletion in the YAC corresponding to these markers. The sequence obtained from PAC 427A4 was searched against the EMBL nucleotide database using the BLASTN program[21]. A complete (100%) match was obtained for the gene encoding the p90 Ribosomal S6 kinase-3 protein. Analysis of the PAC 427A4 sequence showed that it contained the first six exons of the gene (Fig. 1). There were no other coding sequences detected by homology in the PAC sequence. It was estimated on physical data from PACs that the gene for Rsk-3 spans 300kb and is oriented with the 5′ end centromeric and the 3′ end telomeric and is approximately 50 kb from D6S193. During mapping of the marker D6S297 on the three PACs it was found that this is located centromeric to D6S193 rather than telomeric as it is on

the genetic map (Fig. 1), such that the order of the markers are telomere-stSG11162-stSG17631-D6S1585-D6S193-D6S297-centromere. The marker D6S1585 was not available when the genetic map was constructed. There were two ESTs (EST WI 15078 and WI 8751) mapping centromeric to D6S193.

The cDNA for ribosomal S6 kinase-3 (Rsk-3) was isolated in 1995, shown to map to 6q27 by fluorescent *in situ* hybridisation (FISH) and is homologous to two other genes, Rsk-1 and Rsk-2, that map to chromosomes 3p and Xp respectively[5,8]. The Rsks form a unique family of serine-threonine kinases with dual catalytic domains and code for proteins of approximately 90kDa that act downstream of the MAP kinases Erk1 and Erk2 to transduce a variety of signals from the cell surface to the nucleus[22]. The Rsk-2 gene has been shown to be inactivated by mutations in the X-linked disorder Coffin-Lowry syndrome[23]. The p90 Rsk-3 gene fell in the interval of minimal region of deletion on 6q27 and was close to the marker D6S193. An analysis is of the coding sequence for mutations in ovarian tumours was undertaken.

Oligonucleotide primers were designed to amplify by PCR from genomic DNA of ovarian tumours the first six exons for which the intron-exon structure was known from the sequence of the PAC 427A4. A sample of sixteen malignant epithelial ovarian tumours for which allele loss data were already known were selected for analysis[3] by the single strand conformation polymorphism (SSCP) technique[24]. No significant alterations in mobility of bands were identified on SSCP for exons 2-6 in DNA from tumours. It was not possible to amplify exon 1 consistently as

the sequence 5′ of the initiation codon was GC rich due to the CpG island. Primers were then designed to amplify the other exons based on the cDNA sequence of Rsk-3 in the remaining part of the gene inferred both from published exon-intron structures of the catalytic domains of serine-threonine kinases[25] and that of Rsk-2 which is mutated in Coffin Lowry syndrome[23]. Rsk-2 is homologous to Rsk-3 (71%) at the nucleotide level[23]. An additional nine primers (that gave the PCR products of the correct size) were designed to amplify by PCR the subdomain VII and VIII (exon 8) of the first catalytic domain and subdomains I-XI (exons 14-20) of the second catalytic domain[25] from DNA of ovarian tumours. DNA from eight of sixteen malignant tumours amplified by PCR containing putative exons 8 and 14 corresponding to nucleotides 779-921 and 1381-1506 respectively (numbered according to Rsk-3 Acc. No. X85106) demonstrated significant shifts in mobility of bands on SSCP compared to DNA from peripheral blood of each patient (Fig 2A and 2B). The exons 8 and 14 were amplified by PCR from each individual tumour showing a mobility shift on SSCP and cloned into a plasmid vector (pMos*Blue*) and five to ten clones were sequenced. Missense mutations were identified in three tumours within exon 8 and five tumours within exon 14 that predicted to result in a significant substitution of the amino acid (Table 1A, Fig. 3A). Four borderline tumours of the ovary and two benign tumours (for which allele loss data were already available[2]) were then examined together with normal control DNA for alterations in exons 8 and 14. Two tumours were identified which demonstrated shift in mobility of bands (T9 and T37) in exon 8 by SSCP. There was a single base substitution at nucleotide 851 in sample T37 altering the amino acid from methionine to threonine (ATG-ACG) which is a significant mutation

(Table 1A, Fig. 3B) in the 'YMAPE' motif in subdomain VIII and is similar to that observed in the malignant tumour T48 where the methionine was also altered. Two common polymorphisms were identified in exons 8 and 14. In exon 8 the alteration is GCA-GCG at nucleotide 855 (Fig. 3B) that creates a new restriction site for Hae II. In exon 14 the polymorphism was detected at nucleotide 1485 ACA-ACG. None of the significant mutations detected in tumours were observed in the control normal DNA from the same patient. The majority of the amino acid substitutions that were observed in ovarian tumours occurred in conserved residues across species and within the human p90 Rsk family (Fig 2C).

Table 1A

Somatic mutations in the tumour from patients with ovarian cancer

S.No	Age	Stage	Histology	Differentiation	Allele Loss[5]			Ex.	mutation	A.Acid/codon
					149	297	193	264			
48	29	IA	Malignant mucinous	Well	NI	NI	-	+	8	ATG-AGG 851	Met-Arg 226
52	56	II	Malignant serous	Poorly	-	NI	+	+	8	TGG-TGT 903	Trp-Cys 243
46	73	III	Malignant serous	Poorly	NI	+	NI	-	8	TCC-CCC 826	Ser-Pro 218
58	72	IA	Malignant clear cell	Well	NI	+	+			GTG-GCG 866	Val-Ala 231
43	72	III	Malignant serous	Moderate	-	-	+	-	14	ACC-GCC 1489	Thr-Ala 439
8	70	III	Malignant mucinous	Poorly	NI	-	NI	+	14	AAG-GAG 1426	Lys-Glu 418
38	82	Ic	Malignant serous	Moderate	+	NI	NI	NI	14	TTC-TCC 1406	Phe-Ser 411
24	63	I	Malignant clear cell	Well	NI	+	+	+	14	ATC-GTC 1423	Ile-Val 417
37	52	I	Borderline mucinous	-	-	NI	NI	NI	8	ATC-ACC 1424	Ile-Thr 417
9	41	I	Borderline serous	-	NI	NI	NI	MI	8	ACA-GCA 1483	Thr-Ala 437
										GTG-ACG 851	Val-Met 433
										ATG-ACG 851	Met-Thr 226
										GCC-ACC 799	Ala-Thr 209
										TAC-CAC 823	Tyr-His 217

S.No=sample number, Stage=clinical extent of disease according to FIGO, Ex=putative exon of Rsk-3, A.Acid-amino acid, $-all the markers are prefixed by D6S and the data is as previously published[2], MI=microsatellite instability, NI is not informative or unable to amplify, +-allele loss and -=alleles retained, Nucleotide refers to the mutated base (according to Rsk-3 sequence X 85106).

To evaluate the functional status of p90 Rsk-3 an analysis of an unselected sample of ovarian cell lines was undertaken as described previously26 derived from patients with ovarian cancer. Eight ovarian cancer cell lines were analysed for expression of p90 Rsk-3 by Western blotting using specific anti-Rsk-3 antibody. The antibody specifically recognised p90 Rsk-3 which was inhibited in the presence of cognate peptide (data not shown). All the cell lines had p90 Rsk-3 in equivalent amounts except for 59M which was relatively less (Fig. 4A). This suggested that nonsense and truncating mutations in p90 Rsk-3 were unlikely. The MAP kinases Erk-1 and Erk-2 have been shown to bind to p90Rsk-3 and exist in a complex[6,27] and are activated by MEK, the dual specificity kinase. This binding has been shown to be mediated by the C-terminal part of p90 Rsk-3 and it was desirable to establish in cell lines if this function was intact. The p90 Rsk-3 was first immunoprecipitated from all cell lines (following stimulation with EGF) after equalisation for protein content following lysis and then Western blotting was performed with anti-Erk-1 antibody which recognises both Erk-1 and 2 specifically. Erk-1 and 2 were present in all the cell lines suggesting that they are in a complex with p90 Rsk-3 even prior to stimulation with EGF (Fig. 4B). An anti-Erk2 antibody was also used which is specific for Erk-2 and demonstrated that Erk-2 is in complex with p90 Rsk-3 (data not shown). Further, Erk-1 was immunoprecipitated from all the cell lines and then Western blotting was

performed with the antibody against p90 Rsk-3. Fig. 4C demonstrates that p90Rsk-3 was detected in all the cell lines.

Two experiments were performed to examine the catalytic activity of p90 Rsk-3 in all the ovarian cancer cell lines. Firstly, an immune complex *in vitro* kinase assay was performed after immunoprecipitation with anti-Rsk-3 antibody to compare the magnitude of autophosphorylation of p90 Rsk-3 before and after stimulation of the cell lines with EGF. Secondly, an analysis was undertaken of the ability of the immunoprecipitated p90 Rsk-3 to phosphorylate the substrate S6 peptide of the 31kDa (S6) protein that is a component of the 40S ribosomal subunit[5]. In the four cell lines, UC101, OAW42, SKOV-3 and PEO4 there was constitutive activation of the kinase activity of the p90 Rsk-3 as there was autophosphorylation prior to stimulation of cell lines with EGF (Fig. 5A) at basal conditions. In comparison, the basal levels of phosphorylated p90 Rsk-3 in cell lines 59M, 41M, OAW28 and PEO1 was low and comparable to that obtained with HeLa cell line. This is graphically presented in Fig. 5B (results from two experiments) and shows the normal range of phosphorylation after stimulation in cell lines 59M, 41M, OAW28, and PEO1 which is 2-4 fold similar to that obtained with the control cell line HeLa as reported previously[5]. In the four cell lines OAW42, UC101, SKOV-3 and PEO4 the ratios are less than two indicating autophosphorylation of P90 Rsk-3 in the basal state. PEO1 and PEO4 cell lines are derived from the same patient before and after clinical resistance developed to chemotherapy[28].

In the four cell lines OAW42, SKOV3, UC101 and PEO4 the immunoprecipitated p90 Rsk-3 was able to phosphorylate the substrate S6

peptide prior to stimulation with EGF (Fig. 5C, results from 3 experiments). In contrast, in the cell lines 59M, 41M, OAW28, PEO1 and the control cell line HeLa p90 Rsk-3 was unable to phosphorylate the substrate S6 peptide significantly before stimulation with EGF as shown by the sharp increase in catalytic activity after stimulation. The results were also confirmed with another substrate, Kemptide. Kemptide is Leu-Arg-Arg-Ala-Ser-Leu-Gly, available from Sigma Chemical Company, product number K1127 (see also Maller *et al* (1978) *Proc. Natl. Acad. Sci. USA* 75, 248). As a control, the catalytic activity of the related kinase p90 Rsk-2 which is an intermediate in the same signalling pathway as Rsk-3 was examined in the cell lines with constitutive actvation of p90 Rsk-3 and two apparently normal cell lines. The catalytic activity was in the normal range and comparable to that previously reported(Fig. 5D). In order to exclude the possibility that the observed increase in catalytic activity pf p90 Rsk-3 in the four cell lines could be due to activation of an upstream kinase in the Ras-Raf-Mek pathway, the catalytic activity of MEK1 in these cell lines was examined and no evidence of abnormal activity was found.

The identification of constitutive activation of p90 Rsk-3 in four cell lines prompted the examination of the coding sequence for mutations. The coding sequence of Rsk-3 was examined in an identical fashion for mutations as performed for by SSCP. No significant shifts in mobility of bands were detected for any of the exons examined. Consequently, the putative exons 2, 3, 8, 14, 17 and 18 which code for important subdomains in kinases were amplified for all the four cell lines (OAW42, UC101, SKOV3, and PEO4) whose p90 Rsk-3 kinase function was constitutively activated and cell lines 59M and PEO1 where it was

apparently normal. Sequencing of at least five to ten clones was performed for each exon of each cell line. Missense mutations with significant changes in the amino acid residues were identified in all the four cell lines with constitutive activation of p90 Rsk-3 (Table 1b, Fig. 3C). No mutations were detected in the cell lines 59M and 41M. Interestingly, in the cell line PEO4, a 3bp deletion (nucleotide 908-910) was identified which substituted cysteine for phenylalanine and glycine in subdomain IX of the first catalytic domain in the motif 'DWWSFG' (Fig. 2C). PEO which is derived from the same patient had normal sequence in this exon.

Table 1B

Somatic mutations in ovarian cancer cell lines

Cell Line	Ex	Mutation	A. Acid	
UC101	3	GGG-TGG 415	Gly-Trp	81
OAW42	2	AAG-GAG 454	Lys-Glu	94
		ATC-ACC 302	Ile-Thr	43
		CAT-CGT 308	His-Arg	45
OAW42	14	TAC-TGC 1418	Tyr-Cys	45
SKOV3	3	TAC-TTC 437	Tyr-Phe (conservative)	88
SKOV3	14	TGC-CGC 1459	Cys-Arg	429
PEO4	8	del TCG 908-910	Phe & Gly-Cys	245,246

ex=exon, A. Acid=amino acid, the nucleotides refer to the base mutated. del=deletion.

The evidence that Rsk-3 is the gene mutated on 6q27 wherein there is a high frequency of allele loss in ovarian cancer is compelling based on the following data. Firstly, it is the major gene spanning at least 300kb containing approximately 21-22 exons which is within 50kb of D6S193 and is located in the overlap between the minimal regions of deletion as defined originally[16] and subsequently[2]. The difficulty in identifying this gene by directly working on the YAC 171A12 and on cosmids derived from it is underlined by the internal deletion within the YAC. Secondly, mutations have been identified in the coding sequence of Rsk-3 in ovarian tumours and in cell lines which occur in key functional domains of the gene related to its catalytic function (Fig 2C). This is supported by experiments on the catalytic activity of the p90 Rsk-3 in cell lines which has shown that in four of them it is constitutively activated. Thirdly, the identification of mutations in two borderline tumours of the ovary provides strong additional evidence that this gene is involved in the earliest stages of development of ovarian cancer.

The missense mutations detected in cell lines and tumours have so far been in exon 2, 3 and 8 of the first catalytic domain and exon 14 of the second. The first catalytic domain has been shown to be responsible for substrate phosphorylation and bears homology to the cyclic AMP kinase family and the second bears homology to the phosphorylase kinase family[4,25]. Both domains contribute to growth factor stimulated autophosphorylation of p90 Rsk-3. In exon 8, the mutations in T48 and T37, which change methionine in the 'YMAPE' motif to arginine and threonine respectively, are significant mutations and similar to that found in the RET gene in multiple endocrine neoplasia 2B[29] and in the MET gene in hereditary

renal papillary renal carcinomas[30]. This substitution leads to altered substrate specificity and increased transforming ability of the RET protein[31-33]. Although p90 Rsk-3 is an unusual serine threonine kinase in that it has two catalytic domains, it is probable that this mutation would alter substrate specificity as it is the first catalytic domain which is responsible for substrate phosphorylation. The mutations in other exons also occur in amino acids which are highly conserved within the Rsk family across species (Fig 2C). The clustering of mutations in subdomain VIII reflects its role in recognition of peptide substrates that has been substantiated with resolution of the tertiary structure of kinases[25].

In exon 14 which is in the second catalytic domain, the two tumours T43 and T58 have significant substitutions. The substitution of lysine to glutamate (T43) is significant as the latter is a charged amino-acid. This lysine is completely conserved amongst all three members of the p90 Rsk family (Fig 2C). The threonine to alanine mutation (T58) occurs close to the key lysine (K444) which is important in the catalytic activity of Rsk-3. Mutation of lysine (K444) reduces the catalytic activity of p90 Rsk-3 by 50%[4]. The threonine in p90 Rsk-3 is replaced by methionine in Rsk-1 and Rsk-2 suggesting that it might have a unique role functionally in Rsk-3 (Fig 2C). In addition, in tumours T8 and T38 an identical residue isoleucine is mutated to valine and threonine respectively. In general the mutations in exon 14 which corresponds to the first subdomain (by homology with phosphorylase kinase) results in substitutions with less hydrophobic residues. The function of the second catalytic domain in Rsk-3 is primarily to interact with upstream kinases such as Erk-1 and 2 and the region has been mapped to the C-terminal 33 amino acids[6].

However, it also has a potential regulatory role for the kinase function of the first catalytic domain. This is borne out by experiments wherein the first catalytic domain on its own has only 5% of the catalytic activity of the wild type p90 Rsk-3[5].

It is still uncertain whether Erk-1 and Erk-2 are the upstream kinases which phosphorylate p90Rsk-3 as the catalytic activity is not increased significantly upon binding[5,6] as compared to Rsk-1 or 2. However, it is clear that the Erk-1 and 2 kinases exist in a complex with p90 Rsk-3 and are activated by MEK, the dual specificity kinase[6]. The experiments (Fig. 4B) clearly show that in all the cell lines examined this part of the pathway is intact prior to and after stimulation with EGF. The exact physiological substrate for Rsk-3 is as yet unknown. The 31kDa protein (S6) of the 40S ribosomal subunit is phosphorylated *in vivo* by p70 RSK and not by the p90 Rsk family[36]. However, *in vitro* the S6 peptide has been shown to be a good substrate for p90 Rsk-3[5]. The S6 peptide phosphorylation experiments demonstrate and confirm the constitutive activation of p90 Rsk-3 in the four cell lines OAW42, SKOV-3, UC101 and PEO4 (Fig. 5B,C). In each of the cell lines significant mutations have been identified which occur in conserved motifs suggesting possible functional impact. This is supported by analysis of single amino acid substitutions in the catalytic domain of another serine threonine kinase 3pK that increase catalytic activity[35]. In addition, it is unlikely that the upstream activators are responsible for the increased catalytic activity observed in the cell lines as the activity of p90 Rsk-2 which is an intermediate in the same signalling pathway in these cell lines was normal. Further the activity of Mek-1 which signals through the same pathway was

also normal[37]. In addition, mutations in Ras are uncommon in malignant ovarian tumours[15].

None of the mutations detected either in exon 8 or exon 14, are predicted to be inactivating mutations of the gene in relation to catalytic activity or affect binding to Erk1 and 2 as suggested by previous experiments on p90 Rsk-3[4-6]. It has been shown for other serine threonine kinases such as 3pK, Erk1 and Mek1 that mutagenesis of a single amino acid residue is sufficient to render them constitutively active[35]. Mutation of two residues in Mek1 and 3pK further increases the catalytic activity[35]. In the case of p90 Rsk-3, it is possible that two mutations in a single exon within a tumour would alter its function in a similar manner.

This is the first report of a serine threonine kinase that acts downstream of the MAP kinases in the RAS-RAF-MEK-ERK pathway being involved in cancer and at the earliest stage of progression from a benign to malignant state. It is still contentious whether the development of a malignant ovarian tumour progresses from benign to borderline to malignant although pathological examination of tumour specimens often suggest the co-existence of all three stages. This is partly supported by finding mutations in the p53 gene in morphologically normal epithelium adjacent to a malignant field[38,39]. It is now possible to address these questions directly. Although the mutations observed in p90 Rsk-3 do not fit the classical tumour suppressor gene hypothesis in that no inactivating mutations have been found[40], two other receptor tyrosine kinases RET and MET which are implicated in hereditary multiple endocrine neoplasia and familial papillary renal cell carcinoma[29,30] have no inactivating

mutations. Missense mutations that alter function were first reported in the p53 gene on analysis of sporadic tumours together with allele loss at 17p41. The simplest explanation is that mutations in p90 Rsk-3 occur during the process of tumourigenesis, and through a dominant negative effect cause tumour progression. The identification of the involvement of p90 Rsk-3 in ovarian cancer was considerably accelerated by genome sequencing of a defined target area and illustrates the potential advantage of such an approach. Finally, with the rapid development of kinase inhibitors, there is now a clear target, at least in ovarian cancer, for a different therapeutic approach.

Materials and methods

Tumours and cell lines

All the tumours were obtained at the time of surgery of patients with ovarian cancer at the John Radcliffe Hospital, Oxford, UK and were primary specimens. None of the patients had a significant family history of ovarian cancer. The DNA was extracted as previously published and peripheral blood from the same patient was used as control[2]. Eight human epithelial ovarian cancer cell lines were used in this study and were grown in conditions as previously published[26].

PCR, SSCP and Sequencing

PCR was performed according to established methods using a Perkin-Elmer machine. The SSCP gels were run overnight using MDE™ gel

(Flowgen) according to manufacturer's conditions with 10% glycerol at room temperature. Individual exons were amplified from tumours or cell lines by PCR and cloned into pMOS using the pMOS Blue T-vector kit (Amersham) according to manufacturer's conditions. Plasmid minipreps were made using the Wizard Plus Miniprep kit (Promega) and sequenced using the dRhodamine Dye Terminator cycle sequencing kit (Perkin-Elmer) on a ABI Prism 377 DNA sequencer. The sequence was analysed by Sequencher™ 3.0 software (Gene Codes Corporation). Five to ten clones were sequenced in both strands for all exons. On the genomic level, the PAC 427A4 was isolated by PCR and hybridisation screening of the PAC library RPCI-3 (http://bacpac.med.buffalo.edu), fingerprinted and sequenced as described previously[20,42]. The primer sequences were as follows: exon 8

Forward/Reverse 779ATTTCGGCCTGAGTAAGGAGGCC801 & 910CGAAGGACCACCAGTCGGCAC890; exon 14

Forward/reverse 1381CAGTTACACGGGAACAACA1399 & 1490CTTCACGGCATACTCGG 1506.

Cell culture, cell lysis and immunoprecipitation

Cells were grown in 100-mm dishes until they were 90% confluent. After washing the cells once with phosphate buffered saline (PBS), the cells were incubated for 6 hours in Dulbecco's modified Eagle medium (DMEM) supplemented with 0.1% fetal calf serum followed by 15 minutes stimulation with or without EGF (Sigma) at a final concentration of 100ng/ml. The cells were then washed with ice-cold PBS and solubilized on ice in 1 ml of lysis buffer (50mM Tris.Cl, pH 7.4, 1% Triton-x-100, 0.5% Nonidet P-40, 150 mM NaCl, 1mM EDTA, 0.5 mM

EGTA, supplemented with 10μg/ml of pepstatin, aprotinin, leupeptin and trypsin protease inhibitors, 100μg/ml of phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate and 1μM of okadaic acid). The cell lysates were clarified by centrifugation (13 000 rpm, 4°C, 30 minutes) and then quantified for protein content using the BCA assay (Pierce). Equivalent amounts of protein lysate were immunoprecipitated overnight at 4°C with an anti-Rsk-3 specific antibody (Santa Cruz) (1.00 μg/ml) preabsorbed onto 50μl of a 10% solution of protein G (Sigma). Immunoprecipitates were washed three times with lysis buffer, followed by three washes in 0.5M NaCl In addition, for *in vitro* kinase assays the immunocomplexes were washed twice with kinase assay buffer (KA buffer, 30mM Tris.Cl, pH 7.4, 10mM $MgCl_2$, 0.1mM EGTA, 1mM DTT).

Immunoblot Analysis

In order to analyse the relative levels of Rsk-3 and Erk-1 protein expression in the epithelial ovarian cancer cell lines, immunoprecipitation aliquots were resolved on a 8% SDS-PAGE gel in the case of the anti-Rsk-3 immunoblots or 12% in the case of the Erk-1 immunoblots as published previously43. Resolved proteins were transferred onto nitrocellulose and immunoblotted using standard techniques. Rsk-3 (Santa Cruz) and Erk-1 (Signal Transduction Laboratories) specific antibodies were used at a concentration of 1 μg/ml. The bound antibodies were detected by using the respective species specific HRP conjugated antibodies using the Enhanced Chemiluminescence Plus kit (Amersham) according to manufacturer's conditions.

Protein kinase assays

The lysates from the epithelial ovarian cancer cell lines were equalised for protein content prior to immunoprecipitation. The immunecomplex was washed three times with cell lysis buffer followed by three washes in 0.5M NaCl and twice with the kinase assay buffer (KA Buffer: 30mM Tris.Cl, pH 7.4, 10 mM MgCl2, 0.1 mM EGTA, 1 mM DTT, 200μM ATP). *In vitro* autophosphorylation of p90 Rsk-3 was initiated by the addition of 25 μl of KA buffer (containing 200μM ATP) supplemented with 5μCi of [γ-^{32}P]ATP (0.5 μl of a 3000Ci/mmol [γ^{32}P]ATP). The reactions were incubated at 30°C for 10 minutes then terminated by adding 5 μl of boiling 5x SDS sample buffer. After heating at 95°C for a further five minutes, the proteins were resolved by 12% SDS-PAGE. Gels were dried and subjected to autoradiography and Phosphor Imager (Molecular Dynamics) analysis. S6 peptide (RRRLSSLRA, ICRF peptide synthesis Laboratory) was used at a final concentration of 0.5 mg/ml in the S6 substrate phosphorylation assay. The assay was identical to the *in vitro* kinase assay with the exception that the peptide was included. The reaction was terminated by spotting the supernatant onto the phosphocellulose paper (P81 Whatman). After washing thrice with ice-cold 1% phosphoric acid (Sigma), the filters were dried and analysed by Phosphor Imager after a two hour exposure. Goat IgG (1μg/ml) was used in the S6 peptide assay as control. In case of kinase assays for activity of p90 Rsk-2 and Mek1 the experimental condition were as above. A specific C-terminal Rsk-2 and Mek1 antibody (Santa Cruz) were used for these experiments. S6 peptide was the substrate for p90 Rsk-2 and inactive purified Erk1 (New England Biolab) was the substrate for Mek1.

Example 2: Further mutations in the Rsk-3 gene in ovarian tumours

The following mutations were found in the Rsk-3 gene of malignant ovarian tumours:

Tumour	Mutation	Amino acid change
T7	microdeletion	-
T45	microdeletion	-
T61 exon 14	-	-

Example 3: Screening methods

i) ***ELISA-based assay (non-radioactive)***

This technique depends on the generation of antibodies specific for phosphorylated substrate which can be made using methods well known in the art. The substrate used is a short peptide such as Kemptide or a peptide from ribosomal protein S6 which is known to contain target sites for Rsk-3. This peptide is immobilised on, for example, 96- or 384-well microtitre plates. An assay mixture is then added to the wells (containing everything required for phosphorylation to occur - Rsk-3, ATP as phosphate source in appropriate buffer). This is reconstituted *in vitro* with purified proteins or alternatively lysates from Baculovirus-infected insect

(Sf9) cells expressing Rsk-3 are used. The kinase assays are performed in the presence or absence of chemical compounds, peptide mimetics, etc being investigated as inhibitors of the reaction. The wells are then washed out to remove the reaction mixture (leaving the phosphorylated or unphosphorylated substrate in the wells). Each well is then probed using a primary antibody (for example, rabbit) directed against phosphorylated Kemptide or phosphorylated S6 protein (or an antibody that only recognises the unphosphorylated state of the substrate). This antibody should be retained in the wells if the substrate has become phosphorylated. The wells are then probed with a second antibody (covalently coupled to a marker molecule that makes it readily detectable) directed against rabbit antibodies. Commonly used marker molecules include fluorescein or rhodamine dyes (measure fluorescence), and the enzymes peroxidase (horseradish) and alkaline phosphatase (measure biochemically). This method provides ease of detection of phosphorylated and/or unphosphorylated substrate in a high throughput format.

ii) ***Filter' binding assay (radioactive)***

Similar to the ELISA assay described above but not dependent on the availability of antibodies to phosphorylated (or unphosphorylated) substrate. The peptide substrate is immobilised in microtitre dishes as before and the kinase assay is performed as above, this time in the presence of ^{32}P labelled γ-ATP (plus or minus chemical inhibitors). The reaction mixture is washed away and the amount of radioactivity in each well measured, with less radioactivity indicating the presence of an inhibitor of the kinase activity.

This sort of radiolabel incorporation assay may also be performed in such a way that the substrate is not immobilised, the kinase reaction mixture is added and the unincorporated label is removed *via* filtration (hence 'filter' binding assay). The filter can then be counted for radioactivity. Several reactions can be analysed using a large filter.

Example 4: Immunohistochemistry data for Rsk-3 staining pattern in ovarian tumours

Figure 19 shows the results of immunohistochemical analysis of ovarian tumours using an anti-Rsk-3 antiserum.

Example 5: Breast cancer cell lines: Rsk-3 S6 peptide analysis

Figure 20 shows the results of measuring Rsk-3 activity in breast cancer cell lines. *In vitro* kinase assays were carried out using the S6 peptide. The cell lines 2 and 3 do not show any induction of Rsk-3 activity in response to EGF stimulation suggesting possible mutation of Rsk-3 in these cell lines.

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CLAIMS

1. A method for determining the susceptibility of a patient to cancer comprising the steps of

(i) obtaining a sample containing nucleic acid from the patient; and

(ii) contacting the said nucleic acid with a nucleic acid which hybridises selectively to the Rsk-3 gene or a mutant allele thereof, or a nucleic acid which hybridises selectively to Rsk-3 cDNA, or a mutant allele thereof, or their complement.

2. A method of diagnosing cancer in a patient comprising the steps of

(i) obtaining a sample containing nucleic acid from the patient; and

(ii) contacting the said nucleic acid with a nucleic acid which hybridises selectively to the Rsk-3 gene, or a mutant allele thereof, or a nucleic acid which hybridises selectively to Rsk-3 cDNA, or a mutant allele thereof, or their complement.

3. A method of predicting the relative prospects of a particular outcome of a cancer in a patient comprising the steps of

(i) obtaining a sample containing nucleic acid from the patient; and

(ii) contacting the said nucleic acid with a nucleic acid which hybridises selectively to the Rsk-3 gene, or a mutant allele thereof, or a nucleic acid which hybridises selectively to Rsk-3 cDNA, or a mutant allele thereof, or their complement.

4. A method according to any one of the preceding claims wherein the cancer is ovarian cancer or breast cancer or lymphoma.

5. A method according to any one of the preceding claims wherein the sample is a sample of the tissue in which cancer is suspected or in which cancer may be or has been found.

6. A method according to any one of the preceding claims wherein the sample is a sample of ovary and the cancer is ovarian cancer.

7. A method according to any one of the preceding claims wherein the nucleic acid which selectively hybridises to the human-derived DNA of said Rsk-3 gene or the said Rsk-3 cDNA sequence, or a mutant allele thereof, or their complement, further comprises a detectable label.

8. A method according to any one of the preceding claims wherein the nucleic acid which selectively hybridises as said is single-stranded.

9. A method according to any one of the preceding claims wherein the nucleic acid which selectively hybridises as said has fewer than 10000 base pairs when the nucleic acid is double-stranded or bases when the nucleic acid is single-stranded.

10. A method according to any one of the preceding claims wherein the nucleic acid which selectively hybridises as said has fewer than 1000 base pairs when the nucleic acid is double-stranded or bases when the nucleic acid is single-stranded.

11. A method according to any one of the preceding claims wherein the nucleic acid which hybridises as said has from 10 to 100 base pairs when the nucleic acid is double-stranded or bases when the nucleic acid is single-stranded.

12. A method according to any one of the preceding claims wherein the nucleic acid which hybridises as said has from 15 to 30 base pairs when the nucleic acid is double-stranded or bases when the nucleic acid is single-stranded.

13. A method according to any one of Claims 1 to 3 wherein the nucleic acid which hybridises as said comprises a portion of the human-derived DNA of PAC 168L15 or 427A4, or a portion of Rsk-3 cDNA.

14. A method according to Claim 13 wherein the portion is a single-stranded portion.

15. A method according to Claim 14 wherein said portion is capable of amplifying a portion of the Rsk-3 gene or the Rsk-3 cDNA or mRNA in a nucleic acid amplification reaction.

16. A method for determining the susceptibility of a patient to cancer comprising the steps of

(i) obtaining a sample containing protein derived from the patient; and

(ii) determining the relative amount, or the intracellular location, or physical form, of the Rsk-3 polypeptide, or the relative activity of, or change in activity of, or altered activity of, the Rsk-3 polypeptide.

17. A method of diagnosing cancer in a patient comprising the steps of

(i) obtaining a sample containing protein derived from the patient; and

(ii) determining the relative amount, or the intracellular location, or physical form, of the Rsk-3 polypeptide, or the relative activity of, or change in activity of, or altered activity of, the Rsk-3 polypeptide.

18. A method of predicting the relative prospects of a particular outcome of a cancer in a patient comprising the steps of

(i) obtaining a sample containing protein derived from the patient; and

(ii) determining the relative amount, or the intracellular location, or physical form of the Rsk-3 polypeptide, or the relative activity

of, or change in activity of, or altered activity of, the Rsk-3 polypeptide.

19. A method according to any one of Claims 16 to 18 wherein the cancer is ovarian cancer or breast cancer or lymphoma.

20. A method according to any one of Claims 16 to 19 wherein the sample is a sample of the tissue in which cancer is suspected or in which cancer may be or has been found.

21. A method according to any one of Claims 16 to 20 wherein the sample is a sample of ovary and the cancer is ovarian cancer.

22. A method according to any one of Claims 16 to 21 wherein the relevant amount, or intracellular location, of the Rsk-3 polypeptide is determined using a molecule which selectively binds to Rsk-3 polypeptide or a natural variant or fragment thereof.

23. A method according to Claim 22 wherein the molecule which selectively binds Rsk-3 polypeptide or a natural variant or fragment thereof is an anti-Rsk-3 antibody.

24. A method according to any one of Claims 16 to 21 wherein the relevant amount, or intracellular location, of the Rsk-3 polypeptide is determined by assaying or detecting the activity of the Rsk-3 polypeptide.

25. A method according to Claim 22 or Claim 23 wherein the molecule which selectively binds to Rsk-3 comprises a detectable label.

26. Use of a nucleic acid which selectively hybridises to the Rsk-3 gene, or a mutant allele thereof, or a nucleic acid which hybridises selectively to Rsk-3 cDNA, or a mutant allele thereof, or their complement, in the manufacture of a reagent for diagnosing cancer.

27. Use of a molecule which selectively binds to Rsk-3 polypeptide or a natural fragment or variant thereof in the manufacture of a reagent for diagnosing cancer.

28. Use of a nucleic acid as defined in Claim 26 in a method of diagnosing cancer.

29. Use of a molecule which selectively binds to Rsk-3 polypeptide or a natural fragment or variant thereof in a method of diagnosing cancer.

30. A method of determining loss of heterozygosity in a tissue sample, the method comprising the steps of (i) obtaining a sample containing nucleic acid derived from the tissue and (ii) comparing a microsatellite profile of the said nucleic acid with that of a reference (homozygous) tissue, the microsatellite(s) being chosen by reference to the Rsk-3 gene.

31. A method of treating cancer comprising the step of administering to the patient a nucleic acid which selectively hybridises to the Rsk-3 gene or a nucleic acid which hybridises selectively to Rsk-3 cDNA.

32. A method of treating cancer comprising the step of administering to the patient a nucleic acid which encodes the Rsk-3 polypeptide or a functional variant or portion or fusion thereof.

33. Use of a nucleic acid as defined in Claim 26 in the manufacture of a medicament for treating cancer.

34. A method of treating cancer comprising the step of administering to the patient an effective amount of Rsk-3 polypeptide or a fragment or variant or fusion thereof to ameliorate the cancer.

35. Use of Rsk-3 polypeptide or a fragment or variant or fusion thereof in the manufacture of a medicament for treating cancer.

36. A method of treating cancer comprising the step of administering to the patient an effective amount of a compound which inhibits Rsk-3 polypeptide function, or the function of a mutant Rsk-3 polypeptide found in a tumour cell.

37. Use of a compound which inhibits Rsk-3 polypeptide function in the manufacture of a medicament for treating cancer.

38. A method of treating cancer, the method comprising administering to the patient an effective amount of a mutant Rsk-3 polypeptide or fragment thereof, or an effective amount of a nucleic acid encoding a mutant Rsk-3 polypeptide or fragment thereof, wherein the said mutant Rsk-3 is a mutant found in a cancer cell and the amount of said mutant polypeptide or amount of said nucleic acid is effective to provoke an anti-cancer cell immune response in said patient.

39. A cancer vaccine comprising a mutant Rsk-3 polypeptide or fragment thereof, or a nucleic acid encoding a mutant Rsk-3 polypeptide or fragment thereof, wherein said mutant Rsk-3 is a mutant found in a cancer cell.

40. An antibody which reacts with a mutant Rsk-3 polypeptide of fragment thereof, wherein said mutant Rsk-3 is a mutant found in a cancer cell.

41. A nucleic acid which selectively hybridises to a nucleic acid encoding a mutant Rsk-3 polypeptide, wherein said mutant Rsk-3 is a mutant found in a cancer cell.

42. An antibody according to Claim 40 or a nucleic acid according to Claim 41 wherein said mutant Rsk-3 is a mutant found in a cancer cell as disclosed in any of the Examples.

43. A kit of parts comprising a nucleic acid which hybridises selectively to the Rsk-3 gene or a mutant allele thereof, or a nucleic acid which hybridises selectively to Rsk-3 cDNA or a mutant allele

thereof, and means for detecting a mutation in the Rsk-3 gene wherein said mutation is a mutation in Rsk-3 found in a cancer cell.

44. A pharmaceutical composition comprising a gene therapy vector including a nucleic acid which encodes the Rsk-3 polypeptide or a functional variant or portion or fusion thereof and pharmaceutically acceptable carrier.

45. A pharmaceutical composition comprising a gene therapy vector including a nucleic acid which selectively hybridises to the Rsk-3 gene, or a mutant allele thereof, or a Rsk-3 cDNA, or a mutant allele thereof, and a pharmaceutically acceptable carrier.

46. A pharmaceutical composition comprising Rsk-3 polypeptide or a fragment or variant or fusion thereof, and a pharmaceutically acceptable carrier.

47. A nucleic acid as defined in Claim 44 or 45 for use in medicine.

48. Rsk-3 polypeptide or a fragment or variant or fusion thereof, for use in medicine.

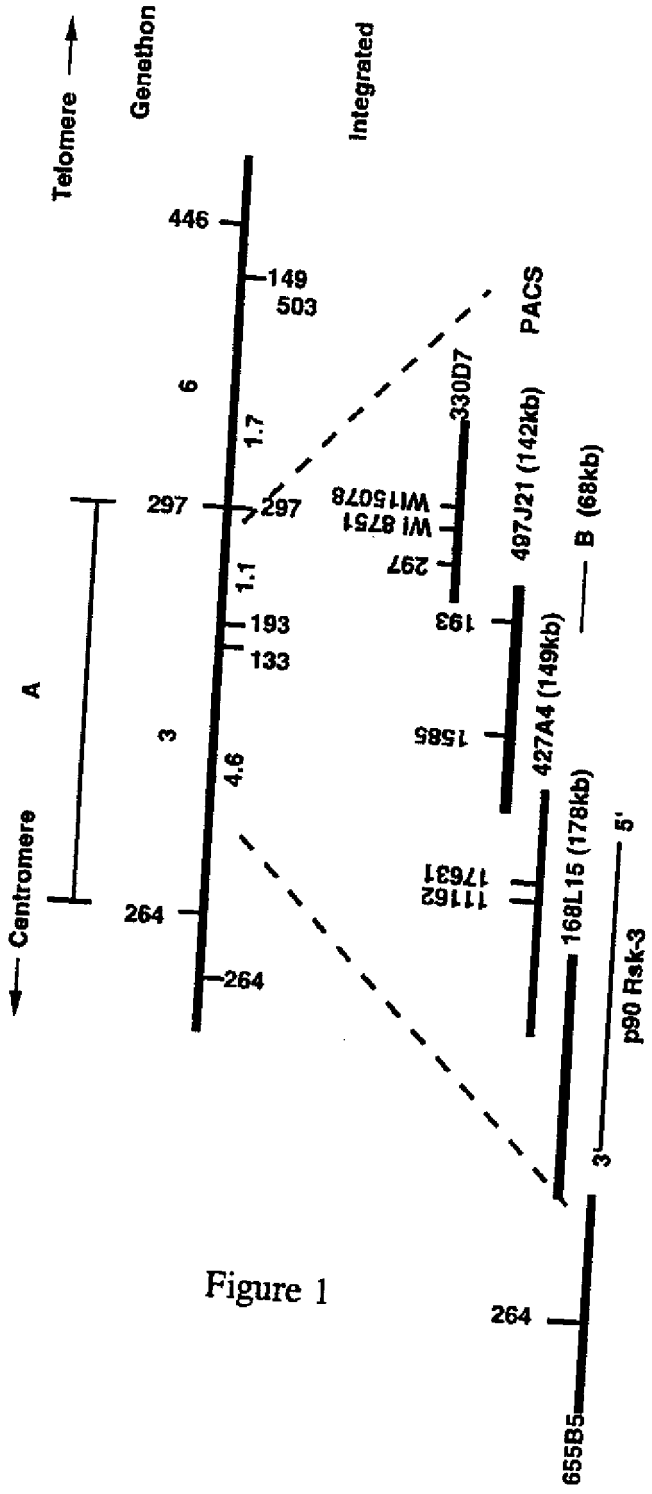

Figure 1

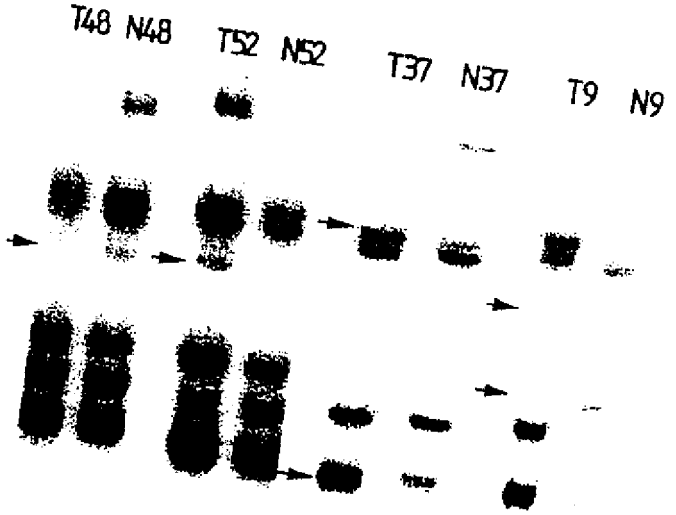

Fig. 2(a)

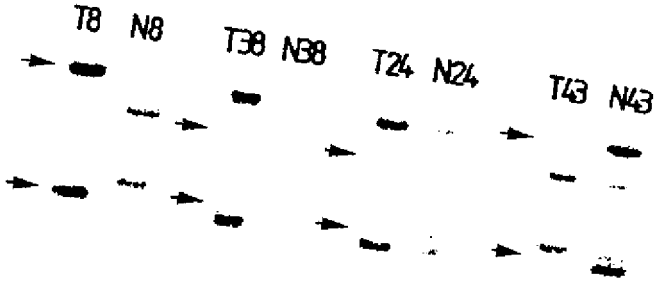

Fig. 2(b)

Figure 2C